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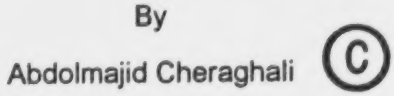
EVALUATION OF 5,6-DIHYDRO PRODRUGS OF 5-ETHYL-2'-DEOXYURIDINE

By

Abdolmajid Cheraghali 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

Edmonton, Alberta

Fall 1994

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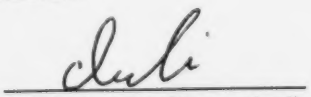

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ز گهواره تا گور دانش بجوی

Search for knowledge since your birth to your death

Prophet Mohammad (S.A.)

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EVALUATION OF 5,6-DIHYDRO PRODRUGS OF 5-ETHYL-2'-DEOXYURIDINE here submitted by ABDOLMAJID CHERAGHALI in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Oct. 6, 1994

to my parents for their best efforts to raise me

to my best friend and beloved wife, Eli, whose support and encouragement were my best support during these years

to my lovely boys, Saeid and Hamed, and to their bright future

Abstract

A series of 5-ethyl-5-halo(Cl,Br)-6-alkoxy(MeO,EtO)-5,6-dihydro-2'-deoxyuridines and 5-bromo-5-ethyl-6-ethoxy-5'(,3')-O-(di)valeryl-2'-deoxyuridine were investigated as potential prodrugs to 5-ethyl-2'-deoxyuridine (EDU) using *in vitro* and *in vivo* models. EDU was rapidly metabolized to 5-ethyluracil and 5-(1-hydroxyethyl)uracil following both iv and oral administration in male Balb-C mice and Spraque-Dawley rats. EDU showed a 49% oral bioavailability in mice following administration of a 200 mg/kg dose. In contrast to the (5R,6R)- and (5S,6S)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro diastereomers of EDU (BMEDU), (5R,6R)- and (5S,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro diastereomers of EDU (CMEDU) did not exhibit *in vitro* anti-HSV activity. However, (+)-*trans*-(5R,6R)-CMEDU showed a very high *in vivo* stability towards nucleoside phosphorylases. In contrast to EDU, 5-(1-hydroxyethyl)-2'-deoxyuridine (HEDU) was detected as a secondary metabolite of BMEDU and CMEDU diastereomers. Compared to EDU, iv injection of [4-^{14}C]-labelled BMEDU diastereomers into mice did not provide significantly ($P > 0.05$) higher radioactivity levels in brain samples. However, iv injection of [4-^{14}C]-labelled (+)-*trans*-(5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-2'-deoxyuridine (BEEDU) provided significantly higher ($P < 0.05$) radioactivity levels in brain samples. BEEDU cleared from blood much faster than BMEDU diastereomers following iv injection into rats. Both the 5'-O-valerate (VBEEDU) and 3',5'-di-O-valerate (DVBEEDU) esters of BEEDU provided significantly higher ($P < 0.001$) radioactivity levels, compared to those after injection of EDU, in brain samples taken following iv injection of the [4-^{14}C]-labelled compounds into mice. However, [4-^{14}C]-DVBEEDU did not provide significantly higher radioactivity levels in brain samples compared

to those after injection of [4-^{14}C]-VBEEDU. In contrast to mice, biliary excretion of (5R,6R)-BMEDU, BEEDU, VBEEDU and DVBEEDU prodrugs was < %4 of the injected dose during a 4 hr period following jugular injection of the [4-^{14}C]-labelled compounds into rats. Both BEEDU and VBEEDU showed similar *in vitro* antiviral activity against different strains of HSV to that of EDU. However, EDU, BEEDU, or VBEEDU did not protect NMRI mice inoculated intracerebrally with HSV-1 or HSV-2. EDU and the 5,6-dihydro prodrugs investigated (BEEDU, VBEEDU) showed substantial accumulation in murine lungs.

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List of abbreviations:

ANOVA	Analysis of variance
AVN-DP	Antiviral nucleoside diphosphate
AVN-MP	Antiviral nucleoside monophosphate
AVN-TP	Antiviral nucleoside triphosphate
AZT	3'-Azido-3'-deoxythymidine
BBB	Blood-brain barrier
BEEDU	(+)-*Trans*-(5R,6R)-5-bromo-6-ethoxy-5-ethyl-5,6-dihydro-2'-deoxyuridine
(5R,6R)-BMEDU	*(+)-Trans*-(5R,6R)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine
(5S,6S)-BMEDU	*(-)-Trans*-(5S,6S)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine
BrDU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BVDU	(E)-5-(2-bromovinyl)-2'-deoxyuridine
C_{max}	Maximum concentration
CDS	Chemical delivery system
CEDU	5-(2-chloroethyl)-2'-deoxyuridine
CF	Correction factor
Cl	Total body clearance
(5R,6R)-CMEDU	*(+)-Trans*-(5R,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine

(5S,6R)-CMEDU	*(+)-Cis*-(5S,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine
CMV	Cytomegalovirus
COMT	Catechol-O-methyl-aminotransferase
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
CT	Computed tomography
DHBG	9-(3,4-Dihydroxybutyl)guanine
DHPG	9-(1,3-Dihydroxy-2-propoxymethyl)guanine
DOPA decarboxylase	Aromatic amino acid decarboxylase
DUMP	Deoxyuridine-5'-monophosphate
DUTP	Deoxyuridine-5'-triphosphates
DVBEEDU	(+)-*Trans*-(5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-3',5'-di-O-valeryl-2'-deoxyuridine
EBV	Epstein-Bar virus
EDU	5-Ethyl-2'-deoxyuridine
EDUDP	EDU-5'-diphosphate
EDUMP	EDU-5'-monophosphate
EDUTP	EDU-5'-triphosphate
EEG	Electroencephalography
ESP	External standard pulse
EU	5-Ethyluracil
FUDR	5-Fluoro-2'-deoxyuridine

GABA-T	Gamma-aminobutyric acid transaminase
GSH	Glutathione
HCMV	Human cytomegalovirus
HEDU	5-(1-Hydroxyethyl)-2'-deoxyuridine
HEU	5-(1-Hydroxyethyl)uracil
HHV	Human herpes virus
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IDU	5-Iodo-2'-deoxyuridine
iv	Intravenous
IVDU	(E)-5-(2-Iodovinyl)-2'-deoxyuridine
LAT	Latency-associated transcripts
MAO	Monoamine oxidase
MRI	Magnetic resonance imaging
MRT	Mean residence time
NIH	National Institute of Health
NDU	5-Nitro-2'-deoxyuridine
P	Partition coefficients
%PB	Percent protein binding
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
po	Oral
SAH	S-Adenosylhomocysteine
SPECT	Single photon emission tomography
$t_{1/2\alpha}$	Half-life of the distribution phase
$t_{1/2\ \beta}$	Half-life of the terminal phase
T_{max}	Time to reach C_{max}
TFT	Trifluorothymidine
TK	Thymidine kinase
TTFD	Thiamin tetrahydrofurfuryl disulfide
VBEEDU	(+)-*Trans*-(5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-5'-O-valeryl-2'-deoxyuridine
VEDU	5'-O-Valeryl-5-ethyl-2'-deoxyuridine
VZV	Varicella-zoster virus

1. Introduction

1.1. Herpes simplex virus (HSV)

HSV belongs to a family of DNA-containing viruses called herpesviridae which is comprised of more than 90 members known to infect many different animal species. So far, seven human herpes viruses are known: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Bar virus (EBV), human herpes virus (HHV) 6 and 7. HSV-1, HSV-2 and VZV, because of similar biological properties such as rapid and lytic spread in cell culture and their ability to establish latent infections in sensory ganglia, are classified in the subgroup of α viruses (Fields et al 1990).

Herpesvirus particles have a diameter from 180 nm (HSV and VZV) to 200 nm (HCMV) (Ginsberg 1988). Complete HSV particles are consist of (Figure 1.1.1) : 1) a cylindrical core structure around which the double-stranded viral DNA is wound (about 75 nm), 2) an icosahedral capsid approximately 85-110 nm in diameter showing 2-3-5-fold symmetry and consisting of 162 capsomers, 3) a granular zone or tegument which surrounds the capsid, and 4) an envelope, which is derived from the host cell as the particles bud from the nuclear membrane (Fields et al 1990). The chemical composition of purified enveloped viral particles is consistent with their morphology. Herpes simplex viron contains 25 to 30 virus-specific proteins (70% of the viron); a large, linear double-stranded DNA molecule (7% of viron); envelope lipid (22% of the viron), which is mainly host-specific phospholipid derived from the nuclear membrane; and small amounts of polyamines (Ginsberg 1988). The structure and molecular composition of capsid has been described recently (Newcomb et al 1993). Within a population of virons, many particles do not possess envelopes, and some are empty capsides (Ginsberg 1988). HSV is relatively sensitive

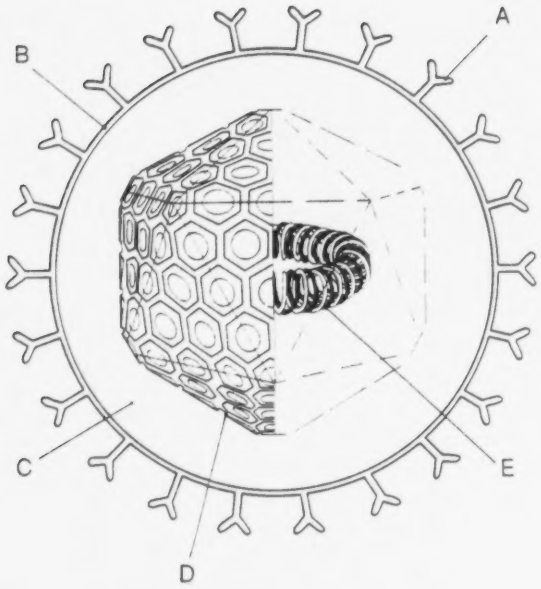


Figure 1.1.1. Structure of HSV. **A** Viral glycoproteins; **B** envelope; **C** Tegument containing specific proteins; **D** Capsid and **E** DNA (from Mindel 1989).

to heat and like other enveloped viruses, HSV is readily inactivated by lipid solvents such as ether, chloroform and alcohol. Different HSVs cannot be distinguished by morphology but can be differentiated by other methods, particularly by serology or by restriction enzyme analysis of their DNA. Two stereotypes of HSV, HSV-1 and HSV-2 and their defined antigenic and biological differences, were first described in 1968 (Nahmias et al 1968). There is about 50 percent homology between nucleic acids in HSV-1 and HSV-2. A variety of methods have been used to differentiate between HSV-

1 and HSV-2, most of which are represented by serologic methods based on antigenic differences. The two viruses also show different biologic properties. For example HSV-2 strains produce macroscopic focal necrotic lesions in the liver of mice on intraperitoneal inoculation, whereas type 1 strains at best only produce a few tiny lesions which are hardly visible to the naked eye (Lopez et al 1986). It was also reported that HSV-2 strains are more virulent in experimental animals. In example, HSV-1 does not grow well in the skin of newborn rabbits and fails to disseminate, whereas HSV-2 disseminates readily to other organs by hematogenous routes. At low virus doses HSV-2 was found to be 1500 times more neurovirulent than HSV-1. These two subtypes can also be differentiated by electron microscopy of infected cells (Lopez et al 1986).

Both HSV-1 and HSV-2 are transmitted by close contact. Type 1 virus is mainly , but not exclusively, transmitted by nongenital contact. HSV-2 is primarily a sexually transmitted disease. The prevalence of HSV-1 antibodies ranges from 50-100% in adult populations while the prevalence of HSV-2 antibodies is generally somewhat smaller but a wider range, from 5-95% (Sakaoka et al 1987).

1.2. Replication of HSV

Infection by HSV begins with attachment of the virus to a cellular receptor, which may differ for HSV-1 and HSV-2. The virus penetrates the cell via fusion of the viral envelope with the plasma membrane (Figure 2.1.2). Transcription of the HSV genome appears to occur in three phases (Fields et al 1990). In phase 1, transcription is mediated by host proteins, either alone or possibly in association with viron proteins. In phase 2, transcription appears to be mediated by proteins which are synthesized

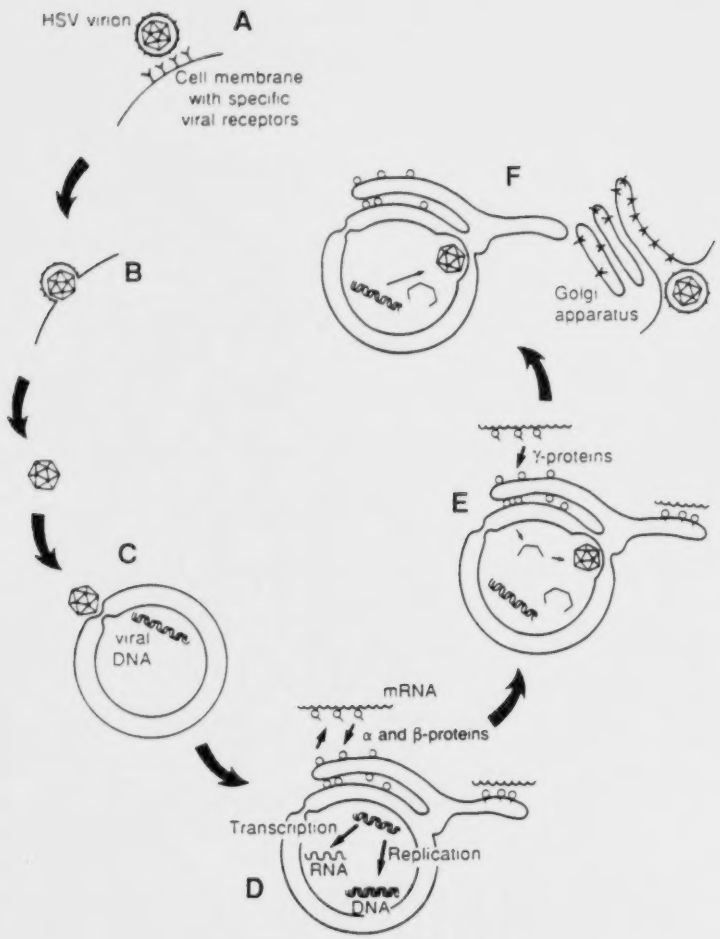


Figure 2.1.2. Viral replication. **A** HSV attaches to a specific viral glycoprotein receptors. **B** Viral envelope merges with cell membrane and viral capsid enters the cytoplasm and is transported to the nucleus. **C** Disassembly of viral capsid and release of viral DNA into the nucleus. **D** expression of α and β proteins. **E** Expression of γ proteins. Formation of new viral genome and capsid. Envelopment of viron as it buds through the inner nuclear membrane into the peri-nuclear space. **F** Transport to Golgi apparatus, where glycosylation of viral proteins occurs. Release into the extracellular space (from Mindel 1989).

after infection, but before the initiation of viral DNA synthesis. In phase 3, transcription is coupled to the initiation of viral DNA synthesis. These three phases of transcription result in the production of mRNAs which eventually lead to the synthesis of three groups of polypeptides: α, β and γ. Synthesis of the viral proteins takes place in the cytoplasm. The transcription of α genes does not require the synthesis of new viral proteins. β proteins include most of the nonstructural proteins, such as those involved in DNA replication, whereas γ proteins include mainly the viral structural polypeptides. The complete cycle of viral replication occurs in approximately 15 hours following infection. However, the processes of replication of virus are inefficient and result in the production of only one infectious virus particle for every 100-1000 which are produced (Fields et al 1990). DNA polymerase and a thymidine kinase (TK) are among the nonstructural proteins which are coded for by HSV DNA. Inhibition of the functions of these enzymes has proved to be an effective approach to chemotherapy in humans (De Clercq et al 1987b).

1.3. Incidence and prevalence of HSV infection

Infection with HSV is extremely common and widespread, occurring world wide with equal incidence in both sexes. While there is no clear-cut seasonal variation in the incidence of HSV infections, some investigators believe that skin infections occur more frequently in the summer months, whereas herpetic keratitis and labialis are more common during the winter months (Fields et al 1990, Whitley et al 1993).

In a study published in 1991, it was estimated that in the United States alone, about half a million people have their first genital herpetic infection in a period of one year, that 10 million people have recurring

genital infections, that there are about 500,000 episodes of herpes keratoconjunctivitis, and that about 720,000 cesarean deliveries are performed annually in order to prevent transmission of the HSV to the newborn. In spite of this, 1800 cases of HSV infection still occur each year in newborns (Roizman 1991). In a recent study, it was reported that in the United States more than 100 million individuals are infected by HSV-1 and at least 40-60 million individuals by HSV-2 (Whitley 1993a).

1.4. Transmission of HSV

Although a variety of animals can be experimentally infected with HSV, man is the only known natural host. No vector has been demonstrated to spread the virus (Fields et al 1990). Transmission of the virus occurs as a result of direct contact with infectious materials. Orofacial infections are generally thought to be most commonly acquired as a result of kissing , and genital herpes simplex infections are acquired as a result of either genital-genital, oral-genital, or anal-genital contact. Other instances of close body contact, as occurs during wrestling, for example, have been associated with outbreaks of mucutaneous HSV infections. Recent reports have documented transmission of HSV by renal transplantation (Gabel et al 1988) and also by dental instruments (Epstein et al 1993).

1.5. Pathology of HSV

Infection with HSV is a common world wide problem. Primary infection with HSV rarely causes significant problems, although widespread involvement in atopic eczema can be life-threatening as it may be associated with encephalitis. It was reported that 20 to 40% of the population at same stage have recurrent orolabial infections with HSV

although in only 1% of the cases this recurrence is severe (Higgins et al 1993). HSV can cause both primary and recurrent infections. However, serology clearly differentiates between primary infection which occurs in non-immune, and recurrent infections, which occur in individuals with a high and stable level of antibody. Antibody surveys indicate that a majority of the adult population have been infected with HSV at some time in their lives. Although primary infections in more than 90 percent of cases are subclinical, primary infections in seronegative adults tend to be more severe than in children (Fields et al 1990).

1.5.1. Primary infections with HSV

The most common clinical manifestation of primary infection with HSV-1 is acute herpetic gingivostomatitis which occurs most frequently in small children (1-3 years old). The lesions tend to heal within 2-3 weeks. However, the recurrent disease usually manifests itself as herpes labialis (cold sores) with typical vesicles at the mucutaneous junctions of lips. Herpetic esophagitis is seen in immunosuppressed patients sometimes together with intraoral herpes infection (Fields et al 1990). Generally, the patient complains of retrosternal pain when swallowing. In patients receiving intensive care, inoculation of HSV from the oral area into the airways or esophagus by tracheal or gastric tubes may occur. HSV can also cause acute necrotizing tonsillitis (Wat et al 1994).

The majority of primary herpes simplex eye infections occurs in adolescents and adults (Fields et al 1990, Reichman 1991). The primary HSV-1 infection in the eye leads to keratoconjunctivitis which might be either unilateral or bilateral. Recurrent eye infection, which in most of cases is unilateral, may exist as keratitis with typical branch-like ulcers and may

last for weeks or even months. Herpes simplex hepatitis is a rare and severe disease with considerable mortality, if untreated. It is usually associated with immunosuppression. It may occur as a part of disseminated herpes simplex , where vesicles on the skin should be of diagnostic help. Even without liver biopsy the disease is strongly indicated by biochemical signs of hepatitis in an immunosuppressed patient with positive HSV cultures from the throat, blood and urine (Gabel et al 1988). Mudido et al reported several cases of hepatitis due to HSV which resulted to death. They also reported pregnancy as a condition that can be predisposed to disseminated HSV infection (Mudido et al 1993).

Disseminated herpes simplex infection, with or without visceral involvement, is seen in severely immunocompromised patients. Myocarditis and jaundice, due to disseminated intravascular coagulation and/or herpetic hepatitis may further complicate the clinical picture. It has also been reported that herpes virus infections are responsible for morbidity and mortality among immnunosuppressed patients (Ljungman 1993). HSV infections were reported to be one of the major causes of death in AIDS patients (Fletcher 1992).

HSV rarely affects the lower respiratory tract. Factors that cause squamous metaplasia of the tracheobronchial tree would predispose the patient to a lower respiratory tract infection with HSV. Such factors include traumatic endotracheal intubation, burns, radiation therapy, cytotoxic chemotherapy and smoking. Patients at risk for HSV infection of the lower respiratory tract include the immunocompromised host such as transplant recipients, patients on cytotoxic chemotherapy, those with AIDS, congenital deficiency of cell-mediated immunity, severe burns, or malnutrition; and

neonates. In bone marrow recipients, HSV pneumonitis occurs within the first 3 weeks after transplantation (Ruben et al 1991).

In patients with atopic eczema or with other dermatoses, HSV infection may involve both affected and nonaffected areas of the underlying skin condition. Death may result from viral dissemination to vital organs or from bacterial superinfections (Fields et al 1990). There are some speculation about the role of HSV in peptic ulcers of the duodenum and in psychiatric and neuralgic diseases. It was reported that HSV can invade the gastrointestinal tract in immunosuppressed mice (Takase et al 1994).

1.5.2. HSV infection of genital tract

HSV infection of genital tract most commonly occurs in adolescents and young adults and is usually due to sexually transmitted HSV-2. Genital herpes simplex is predominately caused by HSV-2, although HSV-1 is also capable of producing the syndrome. An increasing frequency of genital herpes caused by HSV-1 has followed more widespread orogenital sexual habits. In a study involving 1794 patients with HSV genital infection over a 14 year period, it was reported that the proportion of cases that were attributed to HSV-1 increased over the period from approximately 20% to over 40%. It was also reported that HSV-1 infection was more common in the young, in women and as a primary infection (Ross et al 1993). Recent serological surveys employing type-specific antibody assays show that the incidence of HSV genital infections has been underestimated (Kinghorn 1993). These results show a rising prevalence of HSV infections in post-adolescent populations in developed countries. Although the clinical picture of HSV-1 and HSV-2 genital herpes cannot be discerned, HSV-1 tends to cause less recurrence than HSV-2 (Fields et al 1990). The clinical features

of the first episode of genital herpes show marked individual variation in severity and they tend to be more severe in women than in men. A primary genital infection tends to cause the most severe symptoms including dysuria, urinary retention, and sometimes aseptic meningitis. In immunocompromised patients, including those with AIDS, genital herpes may produce excessive lesions, both as primary and as reactivated infection. The large and deep herpetic ulcer of the anal region is a well known finding in AIDS patients (Fields et al 1990).

1.5.3. Herpes infection of neonates

Generalized herpetic disease in newborns is another severe disorder caused by HSV. HSV fetal infection in the first trimenon may result in abortions, stillbirth, prematurity, intrauterine growth retardation and various malformations (Gosch et al 1993). Neonatal HSV infection is primarily the consequence of intrapartum virus acquisition during passage through the birth canal. Infection with HSV in neonates can present in wide spectrum of clinical conditions, from severe generalized disease to subclinical infection. The disease may be manifested by encephalitis or by a disseminated visceral form, with or without involvement of the CNS. In severe infections, it is estimated that 50-65 percent of the untreated cases may be fatal or result in permanent neurological consequences. The estimated incidence of disease is 1/2,500-1/10,000 live births (Peterslund 1991, Whitely 1993b). Most cases have been caused by HSV-2 during birth by contact with herpes lesions in the birth canal and the greatest risk occurs when the mother has primary herpes.

Although useful serological techniques are available for diagnosing primary herpes, strategies to prevent neonatal herpes are limited by the

failure of currently available diagnostic tests to rapidly detect women in labor who are at risk of transmitting herpes and also by transmission of herpes to neonates from asymptomatic mothers. However, cesarean section is indicated in women with clinically apparent genital HSV infection at delivery (Dwyer et al 1993). Although treatment of the HSV-infected neonates with current therapeutic modalities result in localizing of the infection to the skin, eye and mouth and mortality is virtually non-existent, approximately 5% of children are still at risk for a long-term neuralgic consequences (Whitley 1992).

1.5.4. Herpes simplex encephalitis (HSE)

HSV is the most common cause of sporadic, fatal encephalitis in industrialized countries. When untreated, HSE carries a mortality of about 70% with a trail of neurological sequel in 97% of the survivors. Subject to immediate treatment with acyclovir, mortality can be reduced to 25% (Mertens et al 1993). For babies with encephalitis, the mortality has been reduced to approximately 15% and nearly 50% of survivors develop normally 3 years after treatment (Whitely 1993b). HSE is estimated to occur in 1/250,000-1/500,000 persons per year (Skoldenberg 1991). The clinical manifestations of herpesvirus infection of the CNS are indistinguishable from those induced by other viruses. Further characterization of this disease depends on confirmation by virology or histologic findings.

Difficulty arises in distinguishing HSE from other causes of encephalitis, since the clinical presentation, laboratory findings and neurodiagnostic assessment are non-specific (Cameron et al 1992, Koskiemi et al 1991). Methods of detecting HSV-related antigen or antibody in the CSF have suffered from a lack of sensitivity early in the course of the

illness (Aurelius 1993, Goldsmith 1991). HSE occurs predominantly in patients aged 5-30 years old and in those over 50 years old (Peterslund 1991). It may occur as either a primary or a reactivated infection. Beyond the neonatal period, HSE is caused mainly by HSV-1. However, there are several reports of encephalitis caused by HSV-2 (Lortholary et al 1993).

Symptoms of HSE include fever, headache, altered consciousness and mental disorder, bizarre behavior and neurological findings pointing to the fronto-temporal cerebral regions. It was reported that HSE may also cause dyskinesia in survivors (Baxter et al 1994) and that HSE caused severe destruction of the medial portions in the bilateral temporal lobes (Shimada et al 1993). HSE in humans is primarily restricted to the temporal lobe and limbic system. The distribution of lesions suggests that virus enters the brain from a single site and then spreads transneuronally to infect connected structures. Olfactory and trigeminal nerves are two obvious sites of potential viral entry. Trigeminal nerve entry is more likely because it innervates the oral cavity, a common site of initial infection, and the trigeminal ganglion is the most common site of viral latency (Barnett et al 1994). Histological examination of the brains of 8 humans surviving for a period between 4 months and 17 years after acute HSE showed neuronal loss and gliosis that was largely confined to the temporal and frontal lobes. However, there was a widespread persistent inflammatory infiltrate in both the cerebrum and brainstem (Nicoll et al 1993).

1.5.4.1. Clinical presentation of HSE

Symptoms in the initial stage of HSE disease are highly variable. After 1-3 days of general symptoms such as fever, headache and nausea, focal and general neurological; symptoms such as speech, memory or

motor disturbances and seizures, personality changes, disorientation and altered consciousness appear (Aurelius 1993). In the vast majority, but not all, of patients an early examination of the CSF will show a slight to moderate pleocytosis and increased protein (Aurelius 1993).

The clinical features of HSE may be mimicked by a large variety of diseases, including other infections, as well as autoimmune, cerebrovascular and malignant diseases of the CNS. This makes diagnosis of HSE more difficult and it has been reported that in some of the patients initially suspected of having HSE, the diagnosis was not verified (Skoldenberg et al 1984).

1.5.4.2. Diagnosis of HSE

Standard neurodiagnostic procedures include cerebrospinal fluid (CSF) chemistry and cytology, electroencephalography (EEG) and imaging techniques such as computed tomography (CT), single photon emission tomography (SPECT), magnetic resonance imaging (MRI), and also nuclear magnetic resonance (Caudai et al 1994) may be used for diagnosis of HSE. These methods usually yield rapid but non-specific results.

Immunological and serological methods such as detection of HSV-specific IgM, IgA or IgG by immunoassay or western blots and isolation of virus from the brain or CSF are common diagnostic methods for HSV infection. However, these methods are not helpful early in the course of the disease when therapeutic decisions are mandatory (Uren et al 1993, Ho et al 1993, Whitley 1982). Recently, the polymerase chain reaction (PCR) assay has been reported to be the most sensitive test for HSV, Varicella-zoster virus and human papillomavirus infections (Lo et al 1994, Goto 1993). The PCR assay is currently used for typing and quantification of HSV

DNA in CSF of patients with HSE (Ando et al 1993). It was also postulated that a qualitative PCR assay is applicable not only to the diagnosis of HSE but also for monitoring the response to antiviral drugs (Ando et al 1993, Puchhammer-Stockl et al 1993).

Electroencephalogram of HSE patients will usually show abnormalities during the first 2-14 days. In addition to less specific slow-wave changes, patterns with recurring paroxysmal shape-wave ortiphasic complexes with a maximum over one or both temporal lobes are more characteristic. A CT scan of the brain has proved useful in the diagnosis of HSE (Greenberg et al 1990, Hindmarch et al 1986). However, in most cases a CT scan may appear normal. In one study, use of this technique 5 days after the onset of neurological symptoms yielded a sensitivity of 73% and a specificity of 89% (Aurelius 1993). MRI may be useful in the early diagnosis of HSE and is probably more sensitive than CT in the acute stage because of the feasibility of demonstrating edema (Schroch et al 1987, Kohira et al 1992). MRI also renders more detailed information about the brain areas involved. A SPECT with ^{99m}Tc labelled pharmaceuticals, such as ^{99m}Tc-hexamethyl propyleneamine oxime can be useful to discern frontotemporal hyperperfusion in the early stage and hyperperfusion in the later stage of illness (Schmidbauer et al. 1991, Ono et al 1989). Some investigators used radiolabelled antiherpes nucleosides such as (E)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU) for diagnosis of virus infected area in the CNS. However, due to problems such as insufficient penetration of the nucleoside into CNS, results were not very promising (Klapper et al 1988, Iwashina et al 1988, Tandon et al 1988, Cleator et al 1988, Satio 1982).

It appears that isolation of the virus and/or the demonstration of viral antigen is the only specific diagnosis of HSE in early stages. The diagnosis

obtained in this way is unequivocal and also provides informations about the exact strain of the virus. However, the sensitivity of the method to diagnose HSE still is not 100%, as virus replication may take place within a very limited focus (Aurelius 1993). Since earlier antiviral agents used for HSE treatment had many side effects, a specific diagnosis was advocated before initiation of therapy (Whitley et al 1977). However, since acyclovir treatment has been shown to be effective with few side effects, the importance of early treatment for the prognosis has been emphasized and thus led to the opinion that specific diagnosis before treatment is no longer required (De Clercq et al 1987, Aurelius 1993).

1.5.4.3. Treatment and prognosis of HSE

Antiviral treatment trials for HSE have been conducted since the late 1960s. Iodoxuridine was evaluated and found to be both effective and toxic (Alford et al 1975) and cytarabin was found to be no better than placebo (Longson et al 1979). In 1977 vidarabine was shown to reduce mortality of HSE to 4% versus 70% among the placebo recipients (Whitley et al 1977). Acyclovir, administrated iv for 10 days in a dosage of 10 mg/kg four times a day, was found to be superior to vidarabine (Skoldenberg et al 1984, Whitley et al 1986). In another study babies less than one month of age with virologically confirmed HSV infections were randomly assigned to receive either vidarabine or acyclovir for 10 days. However, no difference was found between vidarabine and acyclovir treatment with respect to either morbidity or mortality (Whitley et al 1991).

In the management of the encephalitic patient, corticosteroids are frequently administer in addition to antiviral chemotherapy. Diuretics such as manitol and assisted ventilation are sometimes used in order to control

brain edema (Fishman 1975, Skoldenberg et al 1984, Whitley et al 1986). Although the efficacy of acyclovir in the treatment of HSE has been demonstrated, but more information is needed about the optimal duration of therapy (Nicolaidou et al 1993). There are some recent reported cases focused on the secondary neurologic and mental deterioration of children who had recovered after receiving acyclovir treatment (Neyts et al 1992).

1.6. Latency of HSV

The hallmark of HSV infections is the establishment of the virus in a latent state following the primary disease episode. The mechanisms responsible for establishment, maintenance, and reactivation of latent HSV remain poorly understood.

After attachment and penetration of HSV into the cell, replication takes place in epithelial cells at the periphery. The Virus then infects nerve cell termini and is delivered by axonal transport to the sensory ganglia where it can either replicate or establish latency. In spite of major advances, the mechanism by which HSV avoids elimination from the infected host cell after the acute phase of infection is still poorly understood. In order to achieve a long-term persistence in the infected host, a virus must avoid killing too many host cells, which would result to the early death of the host, and must also avoid elimination by the host immune system (Fraser et al 1993). HSV is thought to be able to achieve persistence by establishing a nonlytic (latent) type of interaction with some of the infected neurons. Following infection, cells in which viral replication occurs are lysed by virus or eliminated by the immune system. Latently infected neurons are not eliminated because neither replication nor clearance by the immune system occurs. Although it is not proved yet, it appears that viral, cellular and

immune factors which are known to regulate HSV immediate early gene expression are the main regulators of the establishment phase of latency (Fraser et al 1993).

Latency and recurrence of HSV occur despite the presence of neutralizing antibodies. Herpes labialis, the so-called fever blister, is the most common type of recurrent HSV lesion, but such lesions may occur in any part of the body (Fields et al 1990, Lycke 1990).

1.7. Reactivation of latent HSV

After establishment of latency, the virus can be reactivated by certain stimuli which stress neuronal cells. Reactivation may lead to replication in the peripheral nervous system, following which virus may be transported either back to the periphery or to the CNS. Replication at the periphery following reactivation is called recurrence, and may once again lead to axonal transport to the sensory ganglia (Fraser et al 1993). Experimental evidence suggests that reactivation is an active process and that viral replication in the neurons is required for recurrence. The fact that TK-negative HSV-1 mutants, which are severely impaired in their ability to replicate in neurons but which do replicate in epithelial cells of the periphery, supports the theory (Stroop et al 1994, Fraser et al 1993). These strains can establish latency in the sensory ganglia, showing that viral replication in neurons is not required for the establishment of latency. However, they do not reactivate in animal models (Stroop et al 1994, Coen et al 1989). It was also reported that HSV-1 is transcriptionally active during latent infection in human peripheral sensory ganglia. Viral gene expression includes the latency-associated transcripts (LATs) which have been linked to the ability of the virus to resume replication and reactivation (Brimanns et

al 1993). Various stimuli are known to induce a recurrence. These are of quite a differing nature, but for individual patients they are often fairly consistent. It is difficult to detect a common denominator for these stimuli. In example with herpes labialis they include emotional stress, sunlight, hyperthermia, food additives, trauma and other factors (Fields et al 1990).

Some authors (Hill 1981) do not agree with the theory of recurrence from ganglia and they believe that the virus is activated by a disturbance in local tissue metabolism caused by interference with the trigeminal root. They developed a theory so-called 'skin trigger theory' of recurrence. According to this theory , HSV is often released from the ganglion to form microfoci of infection in the skin but usually these are eliminated. Physiological changes in the skin, perhaps induced by prostaglandins, occasionally allow a lesion to occur (Hill 1981). However, it is believed that recurrence of the herpetic keratitis happens through a mechanism involving reactivation of latent HSV-1 in the trigeminal ganglia by some inducer (common cold, stress, fever, ultraviolet light, etc.), and transferal of the virus along the trigeminal nerve axon to the cornea (Shimomura et al 1993).

Whether the recurrent herpetic infection has its origin in reactivation of a latent infection localized to peripheral mucutaneous tissues or is a secondary infection spread from an activated latent ganglionic nerve cell infection has been debated for a long time. However, most laboratory and clinical data emphasize that the relevant sites for the latent infection are the sensory and autonomic neurons which also actively participate in the dissemination of the reactivated infection (Fields et al 1990). Although the molecular basis and the mechanisms responsible for the establishment, maintenance and reactivation of the latent HSV infection are not thoroughly understood, it has been reported that the frequency of recurrences after

HSV-2 infections is approximately twice as high as after HSV-1 infection (Reeve et al 1981, Corey et al 1983).

1.8. HSV Immunity

Considering the broad distribution of immunity to herpes simplex virus as determined by presence of significant antibody titers in adult populations and the relative infrequency of classical episodes of primary herpetic infections, the likelihood that most primary infections result in only trivial manifestations of mild illness must be accepted. Infection by HSV results in the production of neutralizing antibodies which are detectable about one week after the appearance of lesions and reach peak titers 4-6 weeks later. The initial antibodies are of the IgM class, but by 4-6 weeks the antibodies are of the IgG class. Once peak titers are reached, the levels of antibodies to the virus remain relatively constant for many years (Lopez et al 1986, Fields et al 1990). These antibodies do not prevent reinfection or the development of recurrent lesions. Recurrent lesions are not associated with an increase in antibody titers. Although the immune responses associated with an initial infection do not prevent reinfection or recurrence, they do alter the course of the disease. Recurrent lesions are rarely associated with systemic symptoms and the lesions are confined to small areas. The lesions of the initial infection resolve slowly and may require 2-3 weeks to heal, while lesions of recurrence or reinfection resolve in 7-10 days. This altered course of the disease is observed across virus types. Persons initially infected with type-1, and subsequently infected with type-2, usually experience less severe illness than persons initially infected with type-2 virus (Lopez et al 1986). Thus, while immunity is not complete, there

appears to be a substantial benefit in reducing the severity of lesions upon reinfection or recurrence (Fields et al 1990).

1.9. Active immunization and vaccines

Viral vaccines fall into two major groups, live attenuated and inactivated. Live attenuated vaccines have generally been utilized in the prevention of those diseases in which systemic invasion of the host with viremia occurs, where only one or few antigenic types of virus exists, where there is stability of antigen, and where immunity is lasting. Example of this type of vaccines are vaccines against smallpox, yellow fever, polio, measles, mumps, and rubella vaccines. In contrast, inactivated vaccines have primarily been used against diseases caused by viruses with numerous antigenic types which stimulate short lived immunity, like some influenza vaccines and rabies vaccine. Neither of these killed vaccines are as effective as the live virus vaccines (Fields et al 1990).

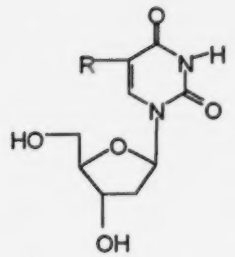
HSV vaccine development has been directed toward the prevention of recurrent infections, using live-attenuated viruses. All attempts to prevent recurrence by immunization in humans with these live viruses have either failed or have been only partially effective (Whitley 1993b). Although subunit vaccines, subunits delivered by live vectors and rationally attenuated vaccines have all been shown to be effective in animal models, they suffer from uncertainties as to the roles of individual genes involved (Farrell et al 1994).

Obviously, patients who suffer from recurrent disease do show the presence of both antibodies and immune lymphocytes. It remains to be determined how in this situation a vaccine which involves the additional administration of a viral antigen, influences the balance of the immune

system and the balance between latency and recurrence. *In vitro* studies have demonstrated that antibodies were not able to stop viral spread whereas virus replication was significantly reduced by the addition of immune effector cells (Kricher 1982). Since 1924 more than 50 HSV vaccines have been tested in various countries and populations. Those vaccines that have undergone vigorous testing failed to protect individuals against infection or to prevent reactivated virus from causing recurrent lesions (Roizman 1991, Banks et al 1994). However, there are some HSV vaccines which are reported to have the potential to prevent the contracting of herpes genitalis (Skinner et al 1992). In general due to presence of epidemologic, serologic and virologic evidence which suggest that HSV might play a role in a variety of human cancers, the possible oncogenic potential of these vaccines should be kept in mind (Dillner 1994).

1.10. Chemotherapy of HSV infections

There are two major classes of antiherpesvirus drugs. The first class which has provided the best clinical outcome is comprised a wide variety of nucleoside analogs. The most used examples of this group are vidarabine, acyclovir, ganciclovir and 5-substiututed-2'-deoxyuridines such as 5-iodo-2'-deoxyuridine (IDU, Iodoxuridine), 5-ethyl-2'-deoxyuridine (EDU), 5-(2-chloroethyl)-2'-deoxyuridine (CEDU), trifluorothymidine (TFT, Trifluridine) and (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) (Figure 3.1.10).



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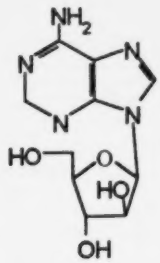
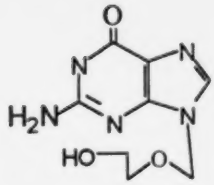
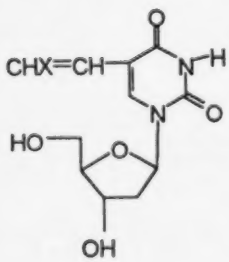


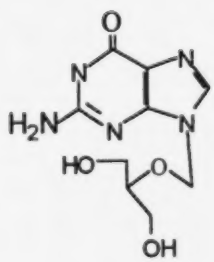
Vidarabine



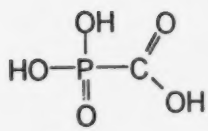
Acyclovir



X= I (IVDU)
X= Br (BVDU)



Ganciclovir



Foscarnet

Figure 3.1.10. Chemical structures for some anti-herpes drugs.

These nucleoside analogs are converted to mono, di, and triphosphate forms by cellular and/or viral thymidine kinase (TK) and it is these phosphorylated forms which are active against virus. The second major class of antiherpesvirus drugs are analogs of pyrophosphate. These include phosphonoacetic acid and phosphonoformic acid (foscarnet) that inhibit viral DNA polymerases directly, evidently by binding to the site involved in releasing the pyrophosphate product of DNA synthesis (Neyts et al 1992, Coen 1991, De Clercq et al 1987b, Keating 1992).

Most antiviral agents which have been developed, or are currently under development, are targeted at one specific step in nucleic acid (RNA or DNA) biosynthesis. In this regard, most nucleoside analogs fall into one of five categories (De Clercq et al 1987b, De Clercq 1990): 1) nucleoside analogs which are dependent upon specific phosphorylation by the virus-encoded TK, 2) nucleoside analogs which are dependent on this enzyme and, moreover, specifically inhibit thymidylate synthase, 3) phosphonylmethoxyalkylpurines and -pyrimidines which are endowed with a broad-spectrum anti-DNA virus activity, 4) acyclic and carbocyclic adenosine analogs which owe their broad spectrum anti-RNA and anti-DNA virus activity to an inhibitory effect on S-adenosylhomocysteine (SAH) hydrolase and 5) 2',3'-deoxynucleoside analogs which are effective against human immunodeficiency virus.

The first group of antiviral nucleosides can be divided into two sub-groups: a) acyclic guanosine analogs such as acyclovir and related compounds and b) 5-substituted-2'-deoxyuridine like EDU, CEDU, IVDU and BVDU. Obviously, these compounds can be expected to be active only against those viruses that encode a virus-specific TK, as do HSV and VZV. Once phosphorylated to their monophosphate (AVN-MP) by the virus-

encoded TK (Figure 4.1.10), the nucleoside analog is subsequently converted to their di- (AVN-DP) and triphosphate (AVN-TP) by cellular enzymes. The active form of all nucleoside analogs which depend for their antiviral activity on the virus-induced TK corresponds to the triphosphate. In this form they may act as competitive inhibitors of the viral DNA polymerase and act as chain terminator. However, EDU is incorporated internally into the DNA strands, that is via an internuclotide linkage (De Clercq et al 1987b).

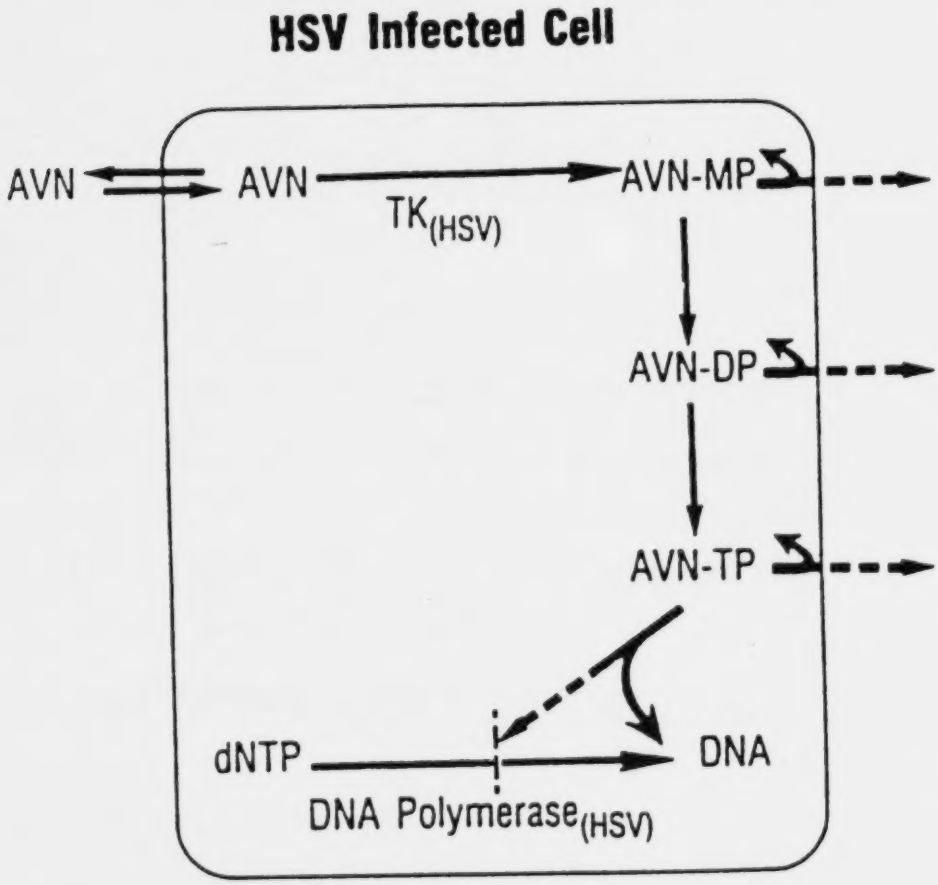


Figure 4.1.10. Mechanism of action of antiviral nucleosides (AVN) which are dependent upon viral TK for their antiviral effects.

The second group of compounds, which are not dependent on TK are targeted at the thymidylate synthase. Some 5-substituted-2'-deoxyuridines which contain a small and electronegative C-5 substituent, such as a fluorine, formyl, nitro, acetylene, cyano, thiocyano or aldoxime group have been recognized as potent inhibitors of thymidylate synthase. 5-Fluoro-2'-deoxyuridine (FUDR), TFT and 5-nitro-2'-deoxyuridine (NDU) are representive examples of this group. It is evident that the activity spectrum of the thymidylate synthase inhibitors extends to TK negative virus strains which are defined as TK negative HSV mutants that may arise during therapy with viral TK dependent drugs.

One reason to investigate nucleosides as potent antiviral agents is that most of these compounds rapidly cross the plasma membrane of the cell by facilitated transport mechanisms (De Clercq et al 1987b), thus gaining rapid entry into the cell. This ability overcomes one of the major barriers for an antiviral agent which must reach the viral infection within the cell to be effective. However, further phosphorylation of antiviral nucleosides by virus TK will increase hydrophilicity of the compounds. The active nucleoside triphosphates, which does not readily leave the cell should remain within the cell long enough to successfully inhibit viral proliferation (Figure 4.1.10).

Phosphonoacetic acid and phosphonoformic acid (foscarnet, Figure 3.1.10) and their analogs have been recognized as a novel class of potent and selective antiviral agents with a very wide spectrum of activity against herpesvirus and other families of viruses (De Clercq et al 1987b). These antiviral compounds, which are not dependent on virus-encoded TK for their activity are active against TK negative HSV. Foscarnet is now the drug of choice for treating both HSV and cytomegalovirus strains resistant to

acyclovir therapy in the clinic (Neyts et al 1992, Verdonck et al 1993). Although there are some reports indicating that lesions of HSV-2 in AIDS patients were resistant to foscarnet, some of the foscarnet-resistant isolates were susceptible to acyclovir *in vitro* (Safrin et al 1994).

In addition, immunotherapy of HSV infections using interleukins is also under investigation (Nakao 1994, De Clercq et al 1987b, Wintergerst et al 1992). It has been reported that extracts of some of the traditional herbal medicines have shown a significant anti-HSV effect (Kurrokawa et al 1993, Elanchezhiyan 1993, Dimitrova et al 1993). It was recently reported that lithium chloride exhibits a substantial anti-HSV-1 effect. It was demonstrated that patients treated with lithium salts for manic depression had a lower incidence of HSV infections. Further investigation confirmed that lithium chloride suppresses herpes virus mRNAs, thereby inhibiting virus replication (Ziaie et al 1994).

1.10.1. Antiviral activity of 5-ethyl-2'-deoxyuridine (EDU)

Among the antiherpes nucleosides, EDU is unique (De Clercq 1985, De Clercq et al 1975, De Clercq 1980a). Its chemotheraputic potential for the treatment of herpesvirus infections was first mentioned in 1966 (Swierkowski et al 1966). EDU did not induce chromosomal aberration (Swierkowska et al 1973) or sister chromatid exchange (Teh et al 1983) in human lymphocytes or fibroblasts. It was subsequently shown that EDU did not induce the release of retrovirus particles from cell lines such as murine Balb-3T3 cells (De Clercq et al 1987a). EDU failed to induce oncogenic RNA (oncorna) viruses in Fisher rat embryo and in Balb/3T3 mouse cells (Gauri 1976). These results are important, because evidence has indicated that chemically induced oncorna viruses, especially as shown for IDU,

ultimately lead to carcinogenesis *in vivo* (Gauri 1976). It has been reported that EDU is as effective as, if not more than, acyclovir for the topical treatment of mucocutaneous HSV-1 and HSV-2 infections in guinea pigs (Spruance et al 1985, Schinazi et al 1985). Controlled double-blind clinical trials have shown the value of EDU in the topical treatment of genital herpes in humans (Sacks et al 1991). EDU is effective against various laboratory strains and clinical isolates of HSV at a minimum effective concentration varying within the range of 1-10 μM, depending on the cell system used (Schinazi et al 1985, Teh et al 1983). It has been shown that EDU was effective against HSE in mice (Davis et al 1978, 1979) and against herpes keratitis (Elze 1979, Imperia et al 1988, Nikoleit 1988, Wassilew 1979).

EDU is inactive against TK negative mutants of HSV (Schinazi et al 1985, De Clercq et al 1980, Cheng et al 1976), which suggests that its antiviral activity is mediated by the virus-encoded TK. In fact, it has been shown that EDU has a much higher affinity for both HSV-1 and HSV-2 encoded TK than for the Hela cell (host) TK (Cheng 1977). It was reported that EDU binds to deoxythymidine kinase about 100-fold weaker than deoxythymidine and the rate of EDU phosphorylation was about the half of the deoxythymidine (Gauri et al 1973). It has been observed that extent of phosphorylation of EDU by HSV-1 and HSV-2-infected cells was 50-fold larger than that by either mock- or TK negative HSV-1 infected cells (De Clercq et al 1980b). However, phosphorylation of EDU-5'-monophosphate (EDUMP) to EDU-5'-diphosphate (EDUDP) and EDU-5'-triphosphate (EDUTP) in HSV-2-infected cells was much less extensive than that by HSV-1-infected cells. This might be due to this fact that HSV-1-encoded, but not the HSV-2-encoded, TK is endowed with thymidylate kinase activity (De Clercq et al 1987a). Compared to other 5-substituted-2'-deoxyuridine-

5'-monophosphate(DUMP) such as 5-fluoro-DUMP, 5-trifluoromethyl-DUMP, 5-nitro-DUMP and 5-formyl-DUMP (Balzarini et al 1982, De Clercq et al 1981), EDUMP is a relatively poor inhibitor of thymidylate synthase (Balzarini et al 1982, Rode et al 1984, De Clercq et al 1987a). The inhibitory effect of EDUMP on thymidylate synthase may be responsible for the inhibition of tumor cell proliferation by EDU (De Clercq et al 1981, Rode et al 1984, Walter et al 1975), but it does not appear to account for its antiviral activity. 5-Alkyl-2'-deoxyuridine-5'-triphosphates (DUTP)s, and particularly EDUTP, can serve as alternate substrates for mammalian, viral and bacterial DNA polymerase (Kowalzick et al 1982,1984). However, EDUTP is a better substrate for HSV-1 and HSV-2 DNA polymerase than for cellular DNA polymerase (Kowalzick et al 1984). It is postulated that incorporation of EDU into DNA result in conformation of less efficient DNA and cause inhibition of viral DNA synthesis which eventually leads to a reduction in virus yield.

The anti-herpes selectivity of EDU is dependent upon its preferential phosphorylation by virus-induced TK and the associated thymidylate kinase, together with the preferential incorporation of EDUTP into viral DNA. The latter process is then followed by a suppression of both viral DNA synthesis and viral progeny formation (De Clercq 1987a, Bernaerts et al 1987).

Unlike 5-bromo-2'-deoxyuridine (BrDU) and IDU, EDU is devoid of mutagenic activity. Although 5-ethyluracil (EU) is readily incorporated into bacterial DNA (Piechowska et al 1965, Pietrzykowska et al 1966), a lack of EDU mutagenicity has been shown in several organisms (Pietrzykowska et al 1966, Swierkowski et al 1966). This lack of EDU mutagenicity compared to IDU might be related to the pK_a for dissociation of its N-3 hydrogen. The

pk_a of IDU is 8.25 and that of EUD is 9.8 (De Clercq et al 1975). This more acidic dissociation constant for the N-3 hydrogen of IDU would increase the chance of faulty base pairing to a base such as deoxyguanosine. It should also be emphasized that EDU is a more "natural" thymidine analog than IDU and that EDU has the same base-pairing properties as thymidine (Shugar 1972). Like thymidine, EDU may undergo photodimerization of the 5,6-olefinic bond followed by photochemical dissociation of the resulting cyclobutane ring to yield 2'-deoxyuridine, a process which would be expected to be nonmutagenic (Krajewska et al 1971). It may be anticipated therefore that substitution of 5-ethyl for 5-methyl in the thymidine of DNA would not lead to any marked changes in the normal conformation of DNA (De Clercq et al 1975).

1.10.2. Other antiherpes nucleoside drugs

Acyclovir and its derivatives are the most used antiherpes drugs for the topical treatment of herpetic keratitis and primary genital herpes, mucocutaneous HSV and VZV infections in immunocompromised patients. Acyclovir is also effective against neonatal herpes and herpetic encephalitis. One of the limitations of acyclovir is its low oral absorption. However, upon oral administration, acyclovir may achieve plasma concentrations that are sufficient to block HSV, but not VZV, replication. Oral administration of acyclovir is not effective against VZV infections, unless it is used at extremely high doses. The poor oral absorption of acyclovir can be overcome by using different types of prodrugs. Acyclovir also has a very low solubility in water (about 0.2% in 25°C). This problem may also be improved using aminoacyl ester prodrugs of acyclovir (Neyts et al 1992, De Clercq 1990, De Clercq et al 1986, Coen 1991, Mindel 1989).

Acyclovir concentrations in CSF are approximately 50% of corresponding plasma concentrations (Morse et al 1993). However, no accumulation of acyclovir in CSF was observed in respect to CSF:plasma concentration ratio.

Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine, DHPG] and **DHBG** [9-(3,4-dihydroxybutyl)guanine] are structurally related to acyclovir (Figure 4.1.10), with similar mechanism of action and spectrum of activity to those of acyclovir. Ganciclovir has two advantages over acyclovir. First, ganciclovir is at least 20-fold more potent than acyclovir in inhibiting the replication of CMV and EBV *in vitro* and second, ganciclovir is about 50-fold more efficacious than acyclovir in the treatment of HSV infection *in vivo*. However, ganciclovir is a bone marrow toxic agent and neutropenia and to a lesser extent, thrombocytopenia are the most frequently observed adverse effects associated with ganciclovir therapy. Other toxic effects of ganciclovir include anemia, eosinopilia, seizures, psychosis, tremor, fever, rash and abnormal liver function. However, recently several less toxic derivatives of ganciclovir were developed (De Clercq 1990, Neyts et al 1993, Mindel 1989). Another major drawback of the clinical use of ganciclovir is its poor bioavailability. In a single-dose study of 10 mg/kg , ganciclovir was 6% bioavailable (Morse et al 1993). CSF concentrations of ganciclovir were reported to be between 24 to 70% of concurrent serum values (Morse et al 1993).

BVDU is a potent and selective HSV-1 and VZV inhibitors. However, HSV-2 and CMV are relatively insensitive to the inhibitory action of BVDU. antiviral activity of BVDU also depends on specific phosphorylation by the virus-encoded TK. BVDU is very well absorbed when given orally. However, BVDU, like most other 5-substitute-2'-deoxyuridine, is a good substrate for

pyrimidine nucleoside phosphorylases which cleave the N-glycosidic linkage. This phosphorolytic cleavage occurs readily *in vivo*, and therefore BVDU is cleared rapidly from the bloodstream (De Clercq 1990, De Clercq et al 1986, Coen 1991, Mindel 1989).

CEDU is another example of a potent and selective inhibitor of HSV and VZV replication. It is about 10 times less active than BVDU *in vitro*, but 10-fold more efficacious than BVDU and acyclovir in the systemic treatment of HSV-1 infections *in vivo*. From the these results, CEDU would appear as a promising agent for the treatment of HSV-1 infections, but its mutagenicity argues against further development of this compound for antiviral therapy purposes (De Clercq 1990, De Clercq et al 1986, Rosenwirth et al 1985).

IDU is a synthetic halogenated pyrimidine nucleoside. IDU is phosphorylated in virus-infected and uninfected host cells by deoxythymidine kinases. The toxicity observed following intravenous use of this drug (mainly bone marrow depression and hepatotoxicity) is probably due to its incorporation into host DNA. IDU established the clinical efficacy, but its toxicity has precluded its widespread use. Its only remaining use is in the treatment of herpetic eye infections. However, even in this context its toxicity has left it as a second-line drug (Mindel 1989).

TFT is another halogenated pyrimidine nucleoside with antiherpes effect. In its mechanism of action, TFT inhibits viral mRNA transcription. Although the drug is active against a variety of DNA viruses, including HSV-1 and HSV-2, its profound bone marrow suppression, due to its incorporation into host DNA and irreversible inhibition of thymidylate synthase, precludes its systemic use. Like IDU its only remaining use is for the management of ocular herpetic infection (Mindel 1989).

Vidarabine is a purine nucleoside analog which has both antiviral and anticancer activity. Vidarabine is active against HSV, but its poor solubility, limited topical absorption, and considerable toxicity has limited its usefulness. Although vidarabine monophosphate is more soluble than its parent compound, it has similar toxic effects as vidarabine has (Mindel 1989).

1.11. Problems regarding drug delivery to brain

Viral infection of the CNS usually causes a severe disease which may culminate in the death of the patient. Several clinical antiherpes drugs are available but most do not provide therapeutic concentration in the brain. In order to reach the viral infected area in the CNS, antiviral drug must cross the blood-brain barrier (BBB). In this section the structure of BBB, problems and currently used approaches regarding delivery of pharmaceutical to the brain will be discussed briefly.

1.11.1. Blood brain barrier (BBB)

Prior to the1980s, the morphological basis of the BBB was a rather controversial issue. However, later investigations revealed that the anatomical structure of the BBB was identified as the endothelial lining of the cerebral capillaries, and most importantly, not the prevascular site (Bodor et al 1983).

There are several ultrastructure differences between systemic and cerebral capillaries which explain the difference in their permeabilities. The main difference is the manner in which endothelial cells in cerebral capillaries are joined. Cerebral junctions are characterized as having tight or close junctions which provide an absolute barrier to some substances.

Structurally, the junctions consist of aligned intramembranous ridges and grooves. The ridges are connected to neighboring ridges by an anastomosing network. The tightness of a junction can be assayed not only by direct structural assessment, but also by measuring ionic conductance and resistance through the junction. These junctions severely restrict the nonspecific transport of materials into the brain (Bodor et al 1983). Lipophilic compounds can readily pass through these phospholipoidal membranes, but hydrophilic compounds and high molecular weight substances are excluded.

The vesicular transport system is also different between the cerebral and systemic capillaries. Vesicular transport is a process for transcellular transport. Cerebral endothelial vesicles are usually uncoated and few in number compared to other systems. This lower vesicular content is another mechanism by which the CNS can limit nonspecific influx.

In addition to structural features, the BBB maintains a number of enzymes which appear to augment barrier function. Since the CNS needs a careful balance between release, metabolism and uptake of neurotransmitters, a high concentration of enzymes such as catechol-O-methyl-aminotransferase (COMT), monoamine oxidase (MAO), gamma-aminobutyric acid transaminase (GABA-T) and aromatic amino acid decarboxylase (DOPA decarboxylase) present in the BBB (Bodor et al 1983).

However, it appears that the BBB barrier is affected not only by structures of cerebral capillaries but there are other factors such as osmosis, ultrafiltration, net charge and/or lipid solubility of the compound, and affinity for transport carrier systems which restrict the entry of some materials and facilitate the entry of others into the brain. Mechanisms

underlying the selectivity of these processes that contribute to the BBB barrier phenomenon have been the subject of several studies, often by measurement of differences in the behavior of closely-related chemically substances. It is known that lipophilic compounds easily cross the BBB (Olendorf 1974, 1978, Levin 1980, Shah et al 1989). However, in addition to lipophilicity, stereospecificity also plays an important role in entry of compound into the brain. For example it was reported that uptake of (-)-D-arabinose into the perfused cat brain was more rapid than that of (+)-D-arabinose. Amino acids also exhibit stereospecificity with respect to their affinity for cerebral transport mechanisms. The uptake of the (-)-enantiomer of tyrosine was more rapid than that of the (+)-enantiomer following iv administration of the amino acids. The brain content of the (-)-isomer after 2 hr was similar to the plasma level whereas (+)-tyrosine did not equilibrate to the same extent. Efflux of compounds from the brain also varies. For example, in one study, it was shown that after subarachnoid injection, (-)-leucine was cleared from the brain within the first hours, whereas the efflux of proline and phenylalanine was considerably slower. Since these amino acids undergo slow metabolism in the brain, their disappearance from the tissue is therefore likely due to efflux rather than catabolism (Mcliwain et al 1985). Although the transport of substances through the normal BBB is restricted by several mechanisms, some conditions such as severe head trauma, CNS disease and infections which lead to encephalitis, and/or meningitis alter permeability of the BBB (Bodor et al 1983).

1.11.2. Drug delivery to the brain

Strategies for pharmaceutical delivery through the BBB may be grouped into three categories: neurosurgically-based, pharmacologically-

based, and physiologically-based approaches. The neurologically-based strategies include the intracarotid injection of drugs, intraventicular drug infusion via an implantable pump, tissue transplantation and the implantation of intratumor polymer matrices. These techniques have been associated with a high incidence of seizure and chronic neuropathologic changes in laboratory animals. However, some of these techniques are particularly useful for the delivery of drugs to a specific site such as intraspinal morphine for analgesia or meningeal delivery of methotrexate for leukemia infiltration of the meninges (Pardridge 1989). Clinical uses of some of these methods are complicated by a high incidence of infection, by obstruction and malfunction of the catheters and devices due to clogging by tissue debris, and by unpredictable release rates of drugs. In addition, other methods such as transient osmotic disruption of the BBB, cerebrospinal fluid perfusion, non-biodegradable or biodegradable polymer delivery systems and direct infusion into a brain tumor utilizing catheters also have been used for delivery of chemotheraputic agents to the brain (Brem 1990).

However, with regard to the drug delivery directly into brain parenchyma via intraventricular delivery systems, there is a functional barrier between the CSF and brain tissue. This barrier arises due to the relative kinetics of bulk flow of CSF through the ventricular spaces versus the rates of drug diffusion into brain parenchyma.

Pharmacologically-based strategies involve the production of liposomes and drug lipidization, or the development of lipid-soluble prodrugs. Liposomes have generally been ineffective for drug delivery through the BBB. This is due to the large size of these structures, which restricts their transport through cellular membranes, including the BBB, and the fact that liposomes usually accumulate in the reticuloendothelial system

(Pardridge 1989, Brewster 1989). A prototypic example of drug lipidization is the conversion of morphine to diacetylmorphine or heroin. The two hydroxyl groups on the morphine nucleus are responsible for the increased polarity of morphine. A general rule is that the placement of a single hydroxyl group on the drug nucleus results in a log order decrease in BBB permeability (Pardridge et al 1979).

Physiologically-based strategies involve the production of chimeric nutrients or chimeric peptides. Chimeric nutrients are drugs that are structurally related to nutrients that normally enjoy carrier-mediated transport through the BBB. Examples of chimeric nutrients are α-methyldopa, L-dopa, α-methylparatyrosine, and phenylalanine mustard or melphalan. These neutral amino acid drugs gain access to brain via carrier-mediated transport through the BBB by the neutral amino acid transporter.

Generally, a compound gains access to the brain via several routes. If the molecule has affinity for one of the carrier systems, it may be transported across the BBB by association with this carrier. A compound with high intrinsic lipophilicity can diffuse passively through the phospholipoidal cell membrane matrix. The ability of a molecule to cross cell membranes is often correlated with its *in vitro* octanol-water partition coefficient. This essentially is a measure of the lipophilicity, and can be correlated with biological effects and with drug permeability through the BBB (Bodor et al 1983, Olendorf 1974, 1978, Hardebo 1979). However, an increase in the ability of a compound to cross membranes is sometimes associated with an increase in undesirable side effects. In addition to passive diffusion and carrier mediation which represent the major routes for influx, there are other less common mechanisms which may also enhance the entry of substrates into the CNS. These include the possible penetration

of substances via the neuron axons via a retrograde mechanism and through those areas of brain which lack a BBB (Bodor et al 1983).

In order to increase the effectiveness of drugs which are active against CNS disease, the pharmacokinetic profile of the agent must be improved and, if necessary, the resident time of the drug in the brain must be increased. If a suitable method were available to do this, it should be possible to deliver an agent specifically to the brain. This specificity should increase the therapeutic index of an agent since not only would the concentration and/or the residence time of the agent increased in the vicinity of the bioreceptor, but of equal importance, the peripheral concentration of the drug, thereby any associated toxicity, would be reduced.

1.12. Use of prodrugs to deliver drugs to the brain

The prodrug concept has been used extensively to optimize the delivery characteristics of many existing drugs and it is often an integral part in the development of some new pharmacologically active agents. The term "prodrug" is used to describe an agent which must undergo chemical or enzymatic transformation to the active or parent drug after administration, so that the metabolic product or parent drug can subsequently exhibit the desired pharmacological response. Therefore, the physiochemical properties of the parent drug and the properties of the site are both critical in predicting whether a prodrug can succeed in the site-specific delivery of the parent drug to a target site.

When the parent or active drug is not fully utilized because of some identifiable barrier or obstacle, the physicochemical properties of the drug can be altered by attachment of a pro-moiety. This allows the prodrug to

bypass the barrier and, once past the barrier, to revert to the parent compound by postbarrier enzymatic or nonenzymatic processes (Figure 5.1.12). Alternatives to cleavage as a method for obtaining activation are enzyme-mediated processes such as phosphorylation.

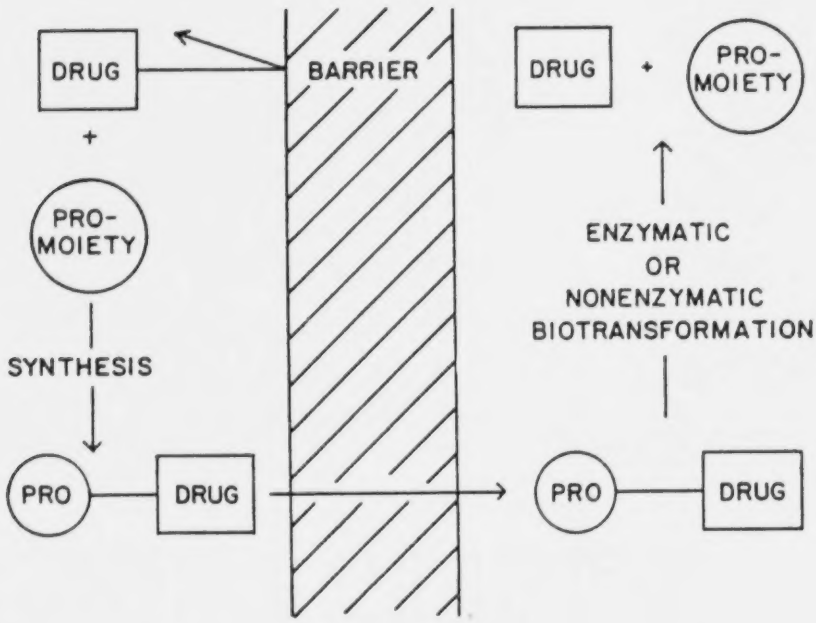


Figure 5.1.12. Improved site-delivery of drugs using prodrug approaches (from Stella 1980).

There are many drugs which have poor access to their site of action. The rate at which a molecule can be transported to an organ is a function of two factors, the blood flow to the organ and the extraction coefficient of the drug by the organ, which is expressed as $Kp=Q \times E$ where Kp is the clearance of the drug from the blood in mL/min, Q is the blood flow to the target organ in mL/min and E is the extraction coefficient or fraction

extracted (Stella 1980). Poor transport to the target organ can be due to two factors. First, the physicochemical properties of the drug molecule in question may cause the molecule to be poorly permeable to some rate-limiting membrane such as the BBB. If this were the case, then E would be small and Kp might be largely determined by the extractability of the drug. On the other hand, if the drug readily permeates the organ, then the blood flow rate may become a limitation. Thus, simply trying to further increase membrane permeabilities of a drug for which E is approximately unity will have no effect on the ability of the drug to reach the target site, since the rate-determining step is blood flow, not extractability. It was reported that for drugs which have octanol/water partition coefficient values greater than 1.0, their rate of BBB transfer will be limited by the blood perfusion rate to the brain. In contrast, the rate of BBB transfer, for compounds having lower P values is limited by the permeability of the BBB. When a drug possesses an intermediate P value, its transfer from blood to brain will be a function of both permeability and perfusion rate. These conclusions are generally limited to small molecules of molecular weights < 450-650, and molecules which do not utilize carrier-mediated mechanism (Bundgaard 1985).

Conversion of a prodrug to active parent drug is a crucial step in the delivery of pharmacologically active agents to the target organ. Low and/or late conversion of prodrug into active drug may provide inadequate delivery of a given drug to the target site using a prodrug approach. For example, prodrugs to norepinephrine are able to reach the site, but their inability to undergo conversion to the parent drug in the target tissue results in the egress of the prodrug from the target site (Stella 1980). Therefore, using increased permeability as the only basis to asses improvement in drug delivery via a prodrug may be an unacceptable or limited criterion. An

alternative criterion is based upon the target organ containing a high level of a particular enzyme which is capable of selectively cleaving the prodrug moiety-drug linkage at that site.

The extraction coefficient is related to the lipophilicity or octanol/water partition coefficient of a compound which is, in turn, related to the ability of a compound to partition into phospholipoidal membranes and, consequently, to organs. Although by increasing the lipophilicity of a compound up to a optimal level, using the prodrug approach, one can increase the entry of a compound into its site of action, this is not a specific process and, in general, all organs are exposed to a greater burden. This is especially important with cytotoxic agents. While increased membrane permeability makes a compound more effective cerebrally there is almost always a disproportionate rise in systemic toxicity. This has severely restricted the clinical efficiency of anticancer prodrugs.

1.12.1. Ester prodrugs

The prodrug approach is a viable method which can be used to enhance the delivery of drugs to the brain. A prodrug is usually designed to provide an improvement in a specific deficient physicochemical property such as water solubility or membrane permeability. Ideally, a prodrug is biologically inactive but reverts to the parent compound *in vivo* which might occur chemically due to some designed instability in the agent or mediated by an enzyme. The aim of these manipulations is to increase the concentration of the active agent at its site of action, thereby, increasing its efficacy. While there are potentially many different types of prodrugs, the majority of the prodrugs are simple esters and amides which readily hydrolyze by ubiquitous hydrolases *in vivo* (Bundgaard 1985). Temporarily

masking the polar groups of a drug enhances the lipophilicity of the drug and its ability to cross membrane is enhanced.

The popularity of using esters as a prodrug type for a drug containing carboxyl or hydroxyl (or thiol) substituents is based primarily on the fact that many tissues, biological fluids or organs are rich in enzymes capable of hydrolyzing esters. The distribution of esterases is ubiquitous, and several types are present in blood, liver and other organs or tissues. In addition, by appropriate esterification of molecules containing a hydroxyl or carboxyl group, it is possible to design derivatives with almost any desired hydrophilicity or lipophilicity as well as *in vivo* stability, the latter being dictated by electronic and steric factors. Accordingly, many hydroxyl or carboxylic acid containing drugs have been modified for a multitude of applications using the ester prodrug approach. The ester prodrug approach has been used to develop prodrugs for many antiviral nucleosides such as acyclovir (Bundgaard 1985, De Clercq 1990), ganciclovir (Powell et al 1991), IDU (Narurkar et al 1989, 1988, Ghosh et al 1991) and also EDU (Martin et al 1989, Keppeler et al 1984). 3',5'-Diesters of FUDR were also studied *in vitro* and *in vivo* as diester prodrugs to FUDR (Kawaguchi et al 1985). Results of *in vitro* and *in vivo* evaluation of these ester prodrugs have shown improved site delivery and/or pharmacokinetic parameters of the parent nucleoside.

1.12.2. Double prodrug

An ideal prodrug should satisfy several criteria at the same time, such as ready conversion to the parent drug, adequate *in vitro* stability and a low toxicity. In many cases it is difficult to design a prodrug with ideal properties in all respects such as a combination of adequate *in vitro* stability

and a facile ability to undergo conversion to the parent drug *in vivo*. The use of cascade latentiation or the double prodrug concept (making a prodrug of a prodrug) has been suggested as a promising mean to overcome various limitations often associated with prodrugs. In the conversion of a double prodrug to a drug, the first step usually involves an enzymatic cleavage and the second step a non-enzymatic hydrolysis that occurs after the first rate-limiting step (Bundgaard 1989). For example, by blocking the free hydroxyl group in the pilocarpine acid esters a double prodrug of pilocarpine is obtained. Such prodrugs were found to be highly stable in aqueous solution. However, it has been demonstrated that in the presence of plasma or rabbit eye tissue homogenate, pilocarpine is formed from the double prodrug in quantitative amounts through a sequential process involving enzymatic hydrolysis of the O-acyl bond followed by the spontaneous lactonization of the intermediate pilocarpic acid ester (Bundgaard 1989).

1.12.3. Chemical delivery systems

A chemical delivery system (CDS) is a popular approach to deliver drugs to the brain. In this delivery system a biologically active compound is linked to a lipophilic dihydropyridine carrier via an ester or amide groups which readily penetrates the BBB. Oxidation of the carrier part *in vivo* to an ionic pyridinium salt prevents its elimination from the brain, while elimination from the general circulation is accelerated (Bodor et al 1981).

After *in vivo* administration of a drug coupled to a CDS carrier, this prodrug is quickly distributed throughout the body, including the brain. The dihydro form is then oxidized to a quaternary pyridinium salt, which because of its ionic-hydrophilic character should be eliminated rapidly from the body, while the BBB should prevent its egress from the brain. Enzymatic cleavage

of the quaternary salt that is locked in the brain will result in a sustained delivery of the drug species, followed by its normal elimination and/or metabolism. A CDS derivtaive of several nucleoside such as 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-(E)-5-(2-iodovinyl)uracil (Morin et al 1993), ribavirin (Bhagrath et al 1991) and 3'-azido-3'-deoxythymidine (AZT) (Brewster et al 1991) synthesized and has been successfully employed for brain delivery of the nucleosides (Bhagrath et al 1991, Brewster et al 1991). An additional example of altered transport that significantly modified the distribution of an agent, due in part to changes in passive prodrug transport, is thiamin tetrahydrofurfuryl disulfide (TTFD), a lipid-soluble prodrug of the quaternary ammonium vitamin, thiamin or vitamin B1. After intravenous administration of thiamin to rats, it has been shown that thiamin was cleared rapidly from blood with a half-life of 35 min. After administration of TTFD, the whole blood thiamin half-life was 200 min and all the thiamin was essentially in the red blood cells. The long half-life of thiamin in whole blood probably results from TTFD rapidly and passively permeating the red blood cell membranes and reacting instantaneously with red blood cell gluthatione (GSH) to release thiamin. Thiamin is thus trapped in the red blood cells and the longer half-life of thiamin in blood simply represents a slow efflux of thiamin from red blood cells (Stella 1980).

1.12.4. 5,6-Dihydro prodrugs of pyrimidine nucleosides

5,6-Dihydro prodrugs are another class of prodrugs to pyrimidine nucleosides. Saturation of the 5,6-olefinic bond of the uracil ring results in a substantial increase in lipophilicity relative to the parent drug. The increased lipophilicity of the 5,6-dihydro prodrug should enhance its diffusion through lipoidal membranes such as the BBB and improve the

cephalic site-delivery of the parent nucleoside. The uracil ring of the 5,6-dihydro prodrug, depending on the nature of substituents of C-5 and C-6, may undergo a rapid regeneration of the 5,6-olefinic bond upon *in vivo* interaction with nuclophilic groups such as thiols. After regeneration of the 5,6-olefinic bond, the more hydrophilic parent nucleoside will be locked inside the cell and exerts its pharmacological effects in a more sustained manner. Application of the 5,6-dihydro prodrug concept to several antiviral and/or anticancer uracil nucleosides has shown promising results with respect to improved site-delivery and pharmacokinetic properties of some uracil nucleosides. It appears that regeneration of the 5,6-olefinic bond may not require any specific enzyme, since it happens in different conditions in *in vitro* and *in vivo* experiments. Therefore, in contrast to the CDS prodrugs, *in vivo* activation of the 5,6-dihydro prodrug may not be a rate limiting step in conversion of prodrug to the parent drug. It is also reported that 5,6-dihydro derivatives of some uracil nucleosides have shown antiviral and/or anticancer activity.

The 5,6-dihydro prodrug approach has been applied to a number of uracil nucleosides and nucleobases. It was reported that 6-alkoxy-5-bromo-5,6-dihydrothymidine derivatives were competitive inhibitors of thymidine kinase obtained from Ehrlich's ascites cells at low concentrations with respect to thymidine (Fouque et al 1974, Teoule et al 1975). 5-Bromo-6-ethoxy-5,6-dihydrothymidine diastereomers are reported to be good candidates for thymidine site-directed isozyme-specific inhibitors of human cytoplasmic thymidine kinase (Hampton et al 1979). 5-Fluoro-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridine diastereomers have been investigated as prodrugs to FUDR (Duschinsky et al 1967). The 5-halo diastereomers possess a number of desirable properties. For example, the 5-bromo, but

not the 5-chloro, diastereomer undergoes regeneration of the 5,6-double bond upon *in vitro* incubation with glutathione (GSH) to give FUDR. The 5-halo diastereomers, unlike FUDR, are stable to pyrimidine phosphorylases. A group of 1-acyl- and 1,3-diacyl-5-alkoxycarbonyl-5-fluoro-6-substituted-5,6-dihydrouracils were also developed as a class of prodrugs to FU (Miyashita et al 1982). Fluorine-18 labelled of the 5-fluoro-6-acetoxy-5,6-dihydrouracil nucleosides were developed and evaluated *in vitro* and *in vivo* as prodrugs to FU (Visser et al 1989). It was also found that some 5-fluoro-6-alkoxy-5,6-dihydro prodrugs of FU blocked incorporation of tritated deoxyuridine into DNA more efficiently than FU (Braakhins et al 1991). 5-Bromo-6-ethoxy-5,6-dihydrouridine and -thymidine derivatives were also evaluated as a class of potential antitumor nucleosides (Bernadinelli 1989). In a recent study synthesis of the 5-bromo-6-alkoxy-5,6-dihydrouracil nucleosides was reported (Samuel et al 1992). 5-Halo-6-methoxy-5,6-dihydro prodrugs of AZT were also employed in order to improve brain delivery of AZT (Wang et al 1994).

These beneficial properties of the dihydrouracil nucleosides prompted us to investigate 5-halo-6-alkoxy-5,6-dihydro analogs of EDU, which are expected to possess a greater *in vivo* stability towards pyrimidine phosphorylases than EDU. The increased lipophilic character of these dihydro derivatives should enhance their ability to cross the BBB, which is essential for the treatment of cephalic viral infections such as HSE.

In this investigation novel 5-halo-6-alkoxy-5,6-dihydro prodrugs, and double prodrugs, to EDU were evaluated using *in vitro* and *in vivo* models.

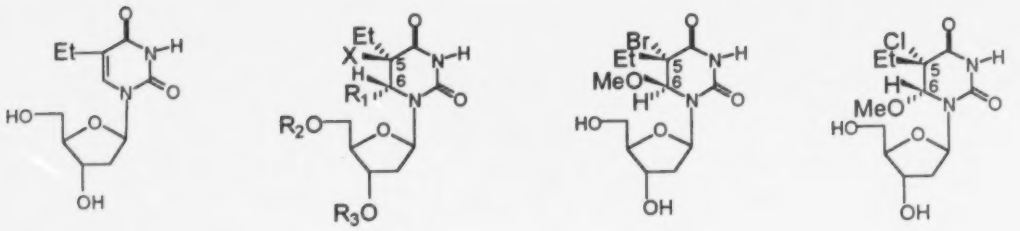
2. Methods and Materials

2.1. Chemicals

5-Ethyl-2'-deoxyuridine (EDU, Figure 1.2.1) and 5-ethyluracil (EU) were purchased from the Sigma Chemical Co. and were used without further purification. 5-(1-Hydroxyethyl)-2'-deoxyuridine (HEDU), 5'-O-valeryl-5-ethyl-2'-deoxyuridine (VEDU), 5-(1-hydroxyethyl)uracil (HEU),*(+)-trans*-(5R,6R)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5R,6R)-BMEDU], *(-)-trans*-(5S,6S)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5S,6S)-BMEDU], *(+)-trans*-(5R,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5R,6R)-CMEDU], *(+)-cis*-(5S,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5S,6R)-CMEDU], (5R,6R)-5-bromo-6-ethoxy-5-ethyl-5,6-dihydro-2'-deoxyuridine (BEEDU), (+)-*trans*-(5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-5'-O-valeryl-2'-deoxyuridine (VBEEDU) and (+)-*trans*-(5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-3',5'-di-O-valeryl-2'-deoxyuridine (DVBEEDU) were synthesized by Dr Rakesh Kumar, Faculty of Pharmacy, University of Alberta. All the 5,6-dihydro prodrugs used in this study were stable in acidic media (pH $\geq$ 2) and upon storage in 4°C for at least 10 weeks. [4-^{14}C]-Labelled [(5R,6R)-BMEDU], [(5S,6S)-BMEDU], BEEDU, VBEEDU and DVBEEDU were synthesized by Kevin W. Morin, Faculty of Pharmacy, University of Alberta. All of the [4-^{14}C]-Labelled compounds had a specific activity of 2GBq (54 mCi)/mmol.

Glutathione (GSH) (reduced, 98%) used for *in vitro* regeneration of the 5,6-olefinic bond experiments and 1-octanol used for measurment of P values were purcahsed from Aldrich Co. 1-Ocatnol was distillated before use. Phosphate buffer solution (pH= 7.00 $\pm$ 0.01) used for measurment of P values was purchased from BDH Inc. Bovine serum albumin (BSA) (clinical reagent grade, 98%+, fatty acid free) used for measurment of percent

protein binding was purchased from ICN Biochemicals Co. Porcine liver esterase (Sigma EC 3.1.1.1, 15.6 mg protein/mL, 185 units/mg of proteins) used for enzymatic hydrolysis of VBEEDU was purchased from Sigma Chemical Co. All solvents used were analytical grade.



EDU **1** **2** **3**

(5S,6S)-BMEDU (5S,6R)-CMEDU

1: (5R,6R)-BMEDU; R_1=MeO, R_2=R_3=H, X=Br

1: (5R,6R)-CMEDU; R_1=MeO, R_2=R_3=H, X=Cl

1: BEEDU; R_1=EtO, R_2=R_3=H, X=Br

1: VBEEDU; R_1=EtO, R_2=Valeryl, R_3=H, X=Br

1: DVBEEDU; R_1=EtO, R_2=R_3=Valeryl, X=Br

Figure 1.2.1. Structures of the 5,6-dihydro derivatives of EDU.

2.2. Animals

Male Sprague-Dawley rats and male Balb-C mice were purchased from the University of Alberta Animal Services Facility. All animals were housed in the Dentistry-Pharmacy Building Animal Services Facility and fed

conventional rodent foods. Mice were housed up to five per group per cage and rats up to three per cage. All studies were performed according to the Canadian Council on Animal Care guidelines, with review and approval by the University of Alberta Health Sciences Animal Welfare Committee. Procedures for the catheterization of the jugular vein and bile duct canulation in rat are described in appendix 1.

2.3. Analytical Systems

2.3.1. High performance liquid chromatography (HPLC)

Quantitative and qualitative analyses of samples for all experiments described were performed using a high performance liquid chromatography (HPLC) system comprised of a Waters Baseline 810 computer program operating on a 486/33 MHz computer, Waters Model 501 pumps, Waters Model U6K injector and Waters Model 486 variable wavelength UV absorbance detector. All separations and quantitative analyses were carried out on a Waters Radial-Pak C18 reverse phase cartridge column (10 μ, 8 mm I.D. × 10 cm Length) at 25°C with UV detection at 230 nm. Acetonitrile and water used as mobile phases were HPLC grade and filtered through a 0.45 μM filter, HA type for water and GV type for acetonitrile, (Millipore) before use.

2.3.2. Analysis of the ^{14}C-labelled samples

All blood and tissue samples taken from animals following iv adminstration of the ^{14}C-labelled compounds [(5R,6R)-BMEDU, (5S,6S)-BMEDU, EDU, BEEDU, VBEEDU and DVBEEDU) were air dried at room temperature for at least three days before combustion using an OX-300

Harvey Biological Material Oxidizer. Tissue sample combustions were carried out using a program that provided a 3 min oxidation at a temperature of 900°C in the combustion zone and 650°C in the catalyst zone. The flow rate for nitrogen and oxygen were 350 mL/min. The instrument was tested before starting combustion of the samples and at the end of each day, using standard ^{14}C-n-hexadecane (Amersham) with known dpm/μL, in order to ensure that the oxidizer was in proper working condition. The [^{14}C]-carbon dioxide produced upon combustion and oxidation of the radioactive tissue samples was trapped in 15 mL of Carbon-14 Cocktail (Harvey Co.). These solutions were then counted using either a Beckman LS 9000 or Mark V series (TmAnalytic) liquid scintillation counter. The total radioactivity present in each of the oxidized samples was calculated from dpm based on a correction factor (CF) for oxidizer using the equations:

Corrected total dpm = total dpm for each sample × CF

CF was calculated using the equations listed below:

CF= dpm added / dpm recovered

dpm added and recovered are as below:

$$\text{dpm added} = \frac{\text{gross cpm of standard added directly to vial - cpm of background}}{\text{counting efficiency}}$$

$$\text{dpm recovered} = \frac{\text{gross cpm of standard recovered after combustion - cpm of background}}{\text{counting efficiency}}$$

Radioactivity in samples was corrected using the H number™ or the external standard pulse (ESP™) as the quench correction method. A

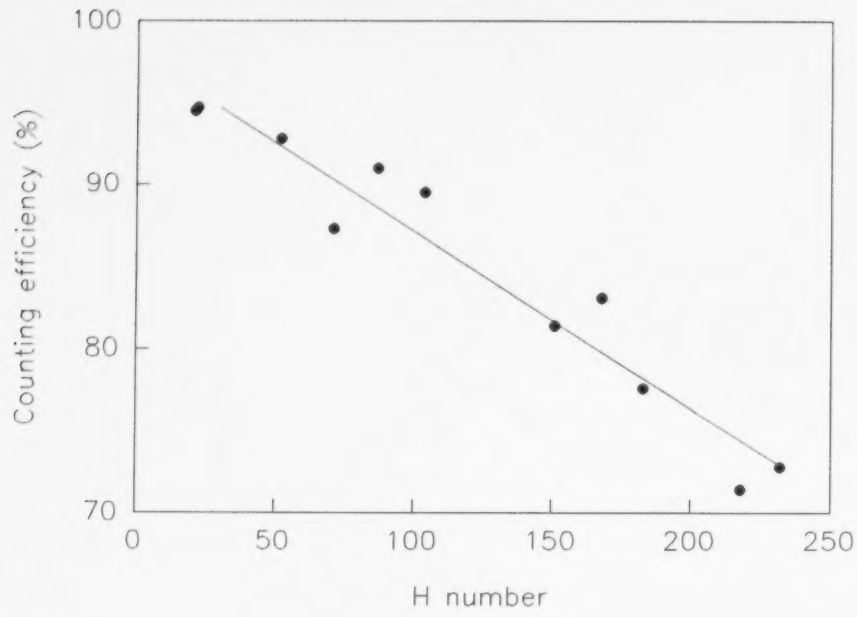
calibration curve was constructed by counting a series of quenched ^{14}C-standard samples (Amersham/Searle). The counting efficiency of each sample was determined using this calibration curve and the activity present in each sample was determined using the dpm=cpm/counting efficiency equation. The calibration curves for H number™ (Y=97.95-0.108X, r=0.967) and ESP™ (Y=98.86-2.8X, r=0.999) are shown in figure 2.2.3.

2.3.3. Analysis of Data

Pharmacokinetic parameters were determined using either the Lagran program (C. Ediss, University of Alberta) or PCNONLIN program (SCI Software). Concentrations or levels of radioactivity of the test compounds in blood or other tissues *vs* time were plotted using SigmaPlot (Jandel Scientific). Statistical significance of the results were measured using either a two way analysis of variance (ANOVA) for independent samples test using the SPSS for Window program (University of Alberta Statistical Counseling Service) or a two tailed *t*-test for independent samples using SigmaPlot program.

2.4. Partition coefficients

Partition coefficients (P) of the compounds (BEEDU, VBEEDU and DVBEEDU) were measured by partitioning the test compound between equal volumes of presaturated 1-octanol and phosphate buffer (pH=7.0). 1-Octanol and buffer were mutually saturated by overnight stirring of equal volumes of octanol and buffer. The two phases were then separated. The concentration of the test compound in the water and octanol phases was determined using HPLC system described in section 2.3.1 and mobile phase consist of water:acetonitrile (80:20, v/v) with flow rate of 2 mL/min.



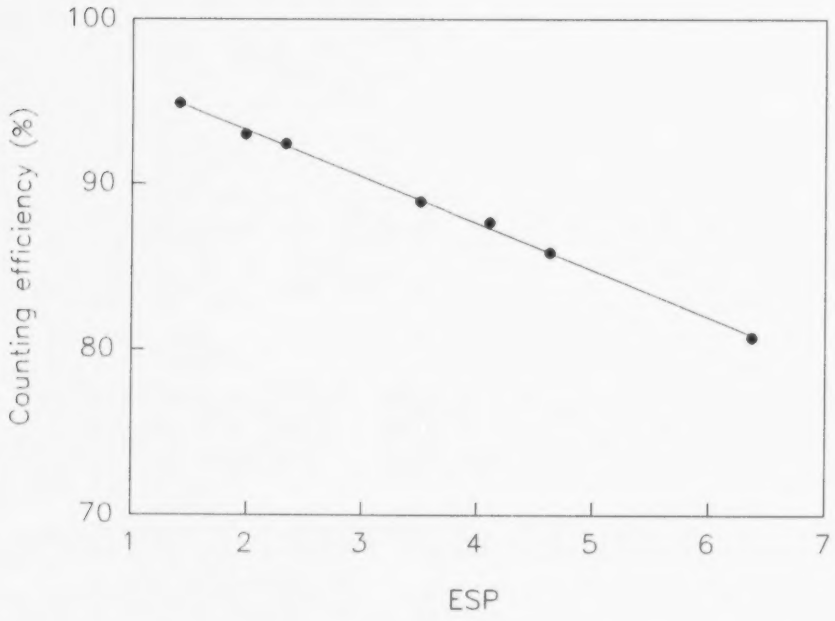


Fig 2.2.3. Calibration curves used for correction of radioactivity present in combusted ^{14}C-labelled samples for H number and ESP methods.

P values were calculated as ratios of the concentration in the octanol to concentration in the water phase ($P= C_{1\text{-octanol}}/C_{buffer}$). A separate calibration curve ($r > 0.955$) was constructed for each test compound to calculate the concentration of the test compound in the aqueous and octanol phases.

2.5. Percent protein binding

The percent protein binding (%PB) of the test compounds EDU, [(5R,6R)-BMEDU], [(5S,6S)-BMEDU], [(5R,6R)-CMEDU], [(5S,6R)-CMEDU] and BEEDU to bovine serum albumin (BSA) was determined by adding different volumes (ranging from 20-300 μL) of a 1×10^{-3}M freshly prepared solution of the test compound in DMSO-water (30:70, v/v) to 0.25 mL of a 1% solution of BSA, previously equilibrated at 37°C. This mixture was agitated using a mechanical shaker for 4 hr at 37°C and the mixture was then filtered through a Centricon-10 (Amicon) (Ghosh et al 1991). The filtrate was analyzed by HPLC using the system described in section 2.3.1. The mobile phase used for quantitation of the samples was isocratic water-acetonitrile (80:20, v/v) with flow rate of 2 mL/min. The percent of compound bound was calculated from the concentration of free compound (C_f) in the filtrate using the following equation:

$\%PB=[(C_t-C_f)/C_t] \times 100$, where C_t is the concentration of the EDU or the 5,6-dihydro compound added to the protein solution.

2.6. *In vitro* stability of the 5,6-dihydro prodrugs

Stability of the 5,6-dihydro substituents *in vitro* for BEEDU was examined in various media by incubation of 100 μL of a 30mM solution (in DMSO:water; 50:50, v/v) of BEEDU with 0.5 mL of either normal saline, rat

plasma, rat whole blood, rat brain homogenate or 2 equivalents of a solution of glutathione (GSH). The brain homogenate was prepared by excising the whole brain from an anesthetized male Sprague-Dawley rat. After rinsing with 15 mL saline, the brain was homogenized in 5 mL of isotonic saline. BEEDU was added to the incubation medium (saline, rat plasma, rat whole blood, rat brain homogenate or GSH) and the mixture was incubated in a shaker-bath at 37°C. Samples were collected after 3, 8, 18, 35, 60 and 120 min incubation times for HPLC analysis using conditions descibed in section 2.5. The % regeneration of the 5,6-olefinic bond present in [(5R,6R)-BMEDU], [(5S,6S)-BMEDU], [(5R,6R)-CMEDU] and [(5S,6R)-CMEDU] was determined upon *in vitro* incubation of 100 μL of a 30mM solution (DMSO:water; 50:50, v/v) with 0.5 mL of either normal saline or 2 equivalents of a solution of GSH. The mixtures were shaken for a period of 24 hr at 37°C and samples from the mixture were analyzed using above mentioned HPLC method.

2.7. Enzymatic hydrolysis of (+)-*trans*-(5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-5'-O-valeryl-2'-deoxyuridine (VBEEDU)

Porcine liver esterase (Sigma EC 3.1.1.1) was used to measure the susceptibility of VBEEDU to enzymatic ester hydrolysis. A 0.4 mL aliquot of the esterase suspension [in 3.2 M $(NH_4)_2SO_4$ solution] was diluted with 0.6 mL of 0.1 M pH 7.0 phosphate buffer. The reaction was initiated by adding 0.03 mmol of VBEEDU in 0.6 mL DMSO:phosphate buffer (50:50, v/v) to the enzyme solution which was previously equilibrated at 37°C. Samples (0.1 mL), which collected at 3, 8, 18 and 35 min post incubation, were poured into 0.1 mL ice-cold acetonitrile to stop the enzymatic reaction. This mixture

was placed in an ice bath for 5 min before centrifugation at 1500 × *g*. The supernatant was then filtered through a 0.45 μ HV filter (Waters Millipore) and an aliquot of the filtrate (20 μL) was subjected to HPLC analysis using conditions which will be described in section 2.13. A blank sample (without esterase) was used as a control to determine whether chemical hydrolysis of the valerate ester occurred during the enzymatic reaction.

2.8. *In vitro* antiviral activity assays

In vitro cytopathic effect (CPE) inhibition assays for HSV-1, HSV-2, HCMV, and VZV and *in vitro* activity of (5R,6R)-BMEDU, (5S,6S)-BMEDU, (5R,6R)-CMEDU, (5S,6R)-CMEDU and EDU, compared to acyclovir, were performed under United States National Institute of Health (NIH) Antiviral Research Branch testing program using the procedures described previously (Kumar et al 1993).

2.9. *In vitro* and *in vivo* anti-HSV activity of 5-ethyl-2'-deoxyuridine (EDU), (+)-*trans*-(5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-2'-deoxyuridine (BEEDU) and VBEEDU

The *in vitro* and *in vivo* antiviral activity of EDU, BEEDU and VBEEDU against several HSV strains in Hela cells, and in NMRI mice inoculated intracerebrally with HSV-1(KOS) or HSV-2(196), were determined at the Rega Institute, Katholieke Universiteit Leuven, Leuven, Belgium using methods described previously (De Clercq et al 1980, 1985, 1989, 1991). To determine the *in vivo* efficacy of the test compounds (EDU, BEEDU and VBEEDU), five twenty-five-day old NMRI mice (weighing 11-13 g) were inoculated intracerebrally with either HSV-1(KOS) at 3 ×

$CCID_{50}$/0.02 mL/mouse or HSV-2(196) at 0.3 × $CCID_{50}$/0.02 mL/mouse dose. The test compound was administered intraperitoneally at various doses (0.20 and 0.78 mmol/kg) twice a day for 5 days, starting on the day of virus infection. Test compounds were dissolved in phosphate-buffered saline (PBS) containing 30% DMSO. Survival rate was calculated for each test compound at the 20th day post inoculation and is reported as the number of live animals per each test group.

2.10. *In vivo* biotransformation of *(+)-trans*-(5R,6R)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5R,6R)-BMEDU], *(-)-trans*-(5S,6S)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5S,6S)-BMEDU], *(+)-trans*-(5R,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5R,6R)-CMEDU] and *(+)-cis*-(5S,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine [(5S,6R)-CMEDU]

The biotransformation of (5R,6R)-BMEDU (5S,6S)-BMEDU (5R,6R)-CMEDU and (5S,6R)-CMEDU, compared to that of EDU, were investigated in rats having an implanted jugular vein catheter. The test compound was injected (100 μL) into the jugular vein using a dose of 0.7 mmol/kg dissolved in DMSO-water (50:50 v/v). Blood samples (200-400 μL) were collected up to 7 hr post injection of the test compound. The catheter was washed after test compound injection and after each blood sampling by injection of 0.4-0.6 mL of heparinized normal saline into the jugular vein catheter. Each blood sample collected was extracted by shaking the whole blood sample with methanol (2 mL) in a mechanical shaker for 15 min. This mixture was centrifuged for 10 min at 2000 ×*g* and the supernatant fraction was then

filtered through a Sep-Pak (C18, Waters Millipore) cartridge. Each Sep-Pak cartridge was preconditioned by washing with methanol (3 mL) and then water (2 mL). The filtrate from the supernatant was dried under a stream of nitrogen gas and the residue obtained was dissolved in methanol (100 μL). A 10 μL aliquot of this solution was then subjected to quantitative HPLC analysis using the HPLC system described in section 2.3.1, using a gradient of acetonitrile (0% for the first 6 min → 15% for the next 11 min → 0% for the rest of the HPLC run) in water (v/v) during a 25 min time interval, with a flow rate gradient of 1.5 mL/min for the first 6 min → 2.5 mL/min for the next 19 min during the separation, with UV detection at 230 nm. The identity of each compound present in the sample was determined by comparison of its retention time to that of an authentic sample. In some instances, the presence of a particular compound was confirmed by spiking an aliquot of the blood sample extract with an authentic sample prior to further HPLC analysis.

2.11. Biotransformation of BEEDU in rats

The biotransformation of BEEDU, relative to EDU, was studied in male Sprague-Dawley rats, 380-400 g in weight, having an implanted jugular vein catheter. BEEDU, or EDU, was injected (300 μL) into the jugular vein catheter using a dose of 0.55 mmol/kg dissolved in DMSO-water (50:50 v/v). Blood samples (200 μL) were collected via the catheter for times up to 4 hr post injection of the test compound and processed as described in section 2.10. The catheter was washed by injection of 0.4 mL of heparinized normal saline into the jugular vein catheter following each dosing and sampling procedure. A 20 μL aliquot of the final solution was then subjected to quantitative HPLC using a gradient of acetonitrile (0% for

the first 6 min → 17% for the next 11 min → 0% for the reminder of the HPLC separation) in water (v/v) during a 25 min time interval, with a flow rate gradient of 1.5 mL/min for the first 6 min → 2.5 mL/min for the next 19 min during the separation, with UV detection at 230 nm. The identity of each compound present in the sample was determined by comparison of its retention time to that of an authentic sample.

2.12. Bioavailability and pharmacokinetic parameters for EDU

The biotransformation and pharmacokinetics of EDU were investigated in mice and rats using EDU doses of 100 mg/kg. EDU was dissolved in 0.1 mL PEG 400:water (30:70, v/v) and injected into the lateral tail vein of mice, or administered orally (po) using a stainless steel gavage syringe. Animals were sacrificed using carbon dioxide at 2, 5, 10, 20, 30, 60, 90, and 120 min post iv injection, and at 5, 10, 20, 30, 45, 60, 90, and 120 min after a po dose. Blood samples (about 0.7 mL) were drawn from the heart. In the oral bioavailability study, animals were deprived of food, but not water, 4 hours before and during the experiment. For the study in rats, EDU (0.1 mL) was injected via an implanted jugular vein catheter. Blood samples (0.2 mL) were collected at 3, 8, 18, 35, 60, 120 and 180 min post injection of EDU. The catheter was washed by injection of heparinized normal saline (0.4 mL) into the jugular vein catheter after dosing and following each sampling procedure.

All blood samples collected in these experiments were mixed with 2 mL of methanol immediately after sampling, and the mixture was shaken for 15 min in a mechanical shaker. The mixture was then centrifuged for 10 min at 2000 x g and the resulting supernatant fraction was filtered through a

preconditioned (section 2.10) Sep-Pak (C18, Waters Millipore) cartridge. After evaporation of the supernatant under a stream of nitrogen gas and dissolving the residue in methanol (400 μL), a 40 μL aliquot of this solution was subjected to quantitative HPLC analysis using the HPLC condition described in section 2.10. The systemic availability (*f*) of the po dose was determined from the ratio of AUC of unchanged drug after po and iv doses based on equation $f = AUC_{po}/AUC_{iv}$.

2.13. Bioavailability and pharmacokinetic parameters for BEEDU and VBEEDU in mice

The bioavailability and pharmacokinetic parameters for EDU, BEEDU and VBEEDU were determined in mice using doses of 0.4 mmol/kg. The test compound was dissolved in 0.1 mL polyethylene glycol (PEG) 400:water (30:70, v/v) and injected into the lateral tail vein of mice, or administered orally (po) using a stainless steel gavage syringe. Animals were sacrificed using carbon dioxide asphyxiation at 2, 5, 10, 20, 30, 60, 90, and 120 min post iv injection, and at 5, 10, 20, 30, 45, 60, 90, and 120 min after a po dose. Blood samples (about 0.7 mL) were drawn via heart puncture and processed as described in section 2.12. In the oral bioavailability study, animals were deprived of food, but not water, 4 hours before and during the experiment.

A 40 μL aliquot of the final solution was subjected to HPLC analysis using the HPLC system described in section 2.3.1. In order to separate VBEEDU from VEDU, BEEDU, EDU and EU a more complex gradient of acetonitrile and water was employed. Detail of the separattion are shown in Table 1.2.13. The systemic availability (*f*) of the po dose was determined

from the ratio of AUC of unchanged drug after po and iv doses based on equation $f = AUC_{po}/AUC_{iv}$.

Table 1.2.13. HPLC conditions for separation of VBEEDU from its metabolites.

time (min)	flow rate (mL)	% acetonitrile	% H_2O
0	1.5	0	100
4	1.5	10	90
14	2.5	20	80
16	3.0	35	65
22	3.5	50	50
24	3.5	30	70
26	3.0	10	90
28	3.0	0	100
30	2.0	0	100

2.14. Biodistribution study

Male Balb-C mice weighing 18-21 g were used for biodistribution studies of [4-^{14}C]-labelled (5R,6R)-BMEDU, (5S,6S)-BMEDU, BEEDU, VBEEDU, DVBEEDU and EDU. The biodistributions of the [4-^{14}C]-labelled test compound were determined after injection of 126 kBq (3.4 μCi), specific activity= 2GBq (54mCi)/mmol, dissolved in 100 μL DMSO-water (50:50 v/v) into the lateral tail vein of mice. Each [4-^{14}C]-labelled test compound was mixed with 0.2 mmol/kg of non-radioactive compound before injection.

Animals were sacrificed and tissues were dissected at 3, 8, 18, 30, 60, 120 min, 12 hr and 24 hr for EDU, (5R,6R)-BMEDU and (5S,6S)-BMEDU and at 3, 8, 18, 30, 60, 120 min time periods post injection for BEEDU, VBEEDU and DVBEEDU. The weights of samples collected from each tissue, which included muscle, bone (femur), brain, lung, large intestine, small intestine, spleen, kidney, urine (content of the bladder), gall bladder, fat and liver, were limited to a maximum 180 mg of wet tissue, or 100 μL of blood and urine, to ensure complete combustion and quantitative trapping of [^{14}C]-CO_2. Samples were air dried at room temperature for at least three days before combustion using an OX-300 Harvey Biological Material Oxidizer using the methods described in section 2.3.2.

The biodistribution of the [4-^{14}C]-BEEDU, [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU were investigated following injection of 126 kBq (3.4 μCi), specific activity= 2GBq (54mCi)/mmol, dissolved in 50 μL DMSO-water (50:50 v/v) of the test compound into jugular vein of mice. The [4-^{14}C]-labelled test compound was mixed with 0.2 mmol/kg of the nonradioactive compound prior to injection and samples were collected and processed using the methods mentioned above.

2.15. Analysis of lung samples after injection of EDU, BEEDU and VBEEDU

The biotransformation and accumulation of EDU, BEEDU and VBEEDU in lung of the male Balb-C mice were determined after iv injection of 0.4 mmol/kg of the test compound into the tail vein. Three mice were used for each experiments. The test compound was dissolved in 0.1 mL of water:polyethylene glycol (PEG) 400 (40:60, v/v). Animals were sacrificed by carbon dioxide asphyxiation at 2, 5, 10, 20, 30, 60, 90 and 120 min post

injection of the test compound. Whole lung and blood samples (about 0.7 mL obtained via cardiac puncture) were collected. The blood samples from each mouse were extracted by shaking the whole blood sample with methanol (2 mL) using a mechanical shaker for 15 min. Lung samples from all three mice were pooled and homogenized in a mechanical homogenizer using 5 mL of normal saline. Either this mixture, or the mixture from blood samples, was centrifuged for 10 min at 2000 × *g* and the supernatant fraction was then filtered through a preconditioned (section 2.10) Sep-Pak™ (C18, Waters Millipore) cartridge. The filtrate from the supernatant was dried under a stream of nitrogen gas and the residue obtained was dissolved in 400 μL methanol. A 40 μL aliquot of this solution was then subjected to quantitative HPLC analysis using the HPLC methods described in section 2.13. The identity of EDU and EU present in the sample was determined by comparison of its retention time to that of an authentic sample.

2.16. Biliary excretion and blood/plasma ratio of [4-^{14}C]-labelled (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU following iv adminstration in rats

Three male Spraque Dawley rats (380-420 g) were used for each experiment. Biliary excretion and blood/plasma ratios for the 5,6-dihydro prodrugs (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU were investigated in anesthetized rats having catheters in the jugular vein for injection of the test compound and blood collection, and in the bile duct for bile collection. The [4-^{14}C]-labelled test compounds [(5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU; 112 kBq (3.0 μCi), specific activity= 2GBq (54mCi)/mmol, dissolved in 200 μL DMSO-water (50:50 v/v)] were

injected into the jugular vein via the catheter. Each [4-^{14}C]-labelled test compound was mixed with 0.2 mmol/kg of non-radioactive compound prior to injection. The catheter was washed with 0.4 mL of heparinized saline after injection of the test compound and after collection of each blood sample. Blood samples (two of 0.1 mL) and bile samples were collected at 3, 8, 18, 35, 60 120, 180, and 240 min post injection. The excretion rate of radioactivity via bile was calculated using the ΔX_b / Δt equation, where ΔX_b is the amount of radioactivity excreted in bile divided by the time period. These data were then plotted against the mid-point sampling time.

Blood samples were collected in heparinized tubes and one blood sample from each time period was centrifuged at 1500 $\times g$ to separate the plasma from the blood cells. All blood and tissue samples were air dried at room temperature for at least three days before combustion using an OX-300 Harvey Biological Material Oxidizer using the method described in section 2.14.

3. Results

3.1. Partition coefficients and percent protein binding

Partition coefficients (P), molecular weights and percent binding to bovine serum albumin (BSA) of EDU, (5R,6R)-BMEDU, (5S,6S)-BMEDU, (5R,6R)-CMEDU, (5S,6R)-CMEDU, BEEDU, VBEEDU and DVBEEDU are summarized in Table 1.3.1.

The 5,6-dihydro derivatives showed significantly higher P values (0.4-11.5) compared to that of EDU (0.08). However, among the prodrugs with unprotected hydroxyl group(s), (5S,6S)-BMEDU and (5R,6R)-CMEDU showed the highest P values (2.5) whereas (5S,6R)-CMEDU exhibited the lowest P value

Table 1.3.1. Partition coefficients (P), molecular weights and percent protein binding (%PB) of the 5,6-dihydro prodrugs of EDU.

Compound	%PB	P[a]	Log P	MW[b]
EDU	7 ± 2.2	0.08[c]	-1.1	256.3
(5R,6R)-BMEDU	19 ± 6.6	1.9[c]	0.3	367.2
(5S,6S)-BMEDU	14 ± 2.5	2.5[c]	0.4	367.2
(5R,6R)-CMEDU	22 ± 5.3	2.5[c]	0.4	322.7
(5S,6R)-CMEDU	11 ± 1.3	0.4[c]	-0.4	322.7
BEEDU	20 ± 3.6	1.1	0.04	381.2
VBEEDU	ND[d]	11.5	1.1	465.3
DVBEEDU	ND	ND	-	549.2

[a]Concentration in 1-octanol/phosphate buffer
[b]Molecular weight
[c]Data from Cheraghali et al 1994
[d]Not determined.

(0.4). Esterification of the C-5' hydroxyl group of BEEDU gave VBEEDU which resulted in a one log order increase in lipophiliicty. Due to the very high lipophilicity of DVBEEDU, measurement of its P value was not feasible.

In comparison to EDU which showed 7% binding to BSA, the 5,6-dihydro derivatives showed percent protein binding, ranging from 11-22%, to BSA (Table 1.3.1). Among the prodrugs, (5S,6R)-CMEDU showed the lowest (11%) and (5R,6R)-CMEDU showed the highest (22%) binding to BSA.

3.2. Biotransformation of (5R,6R)-BMEDU, (5S,6S)-BMEDU, (5R,6R)-CMEDU, (5S,6R)-CMEDU and EDU in rats

Results from biotransformation studies of (5R,6R)-BMEDU, (5S,6S)-BMEDU, (5R,6R)-CMEDU and (5S,6R)-CMEDU, compared to that of EDU,

Table 2.3.2. Pharmacokinetic parameters for EDU and its 5,6-dihydro derivatives followingiv administration of a 0.7 mmol/kg dose into rats.

Compound injected	$AUC^{a,b}$ (μmol.hr.mL^{-1})			Cl^a (L.hr^{-1})	$t_{1/2}{}^a$ (hr)
	Prodrug	EDU	EU		
EDU	-	0.33	0.44	5.1	0.69
(5R,6R)-BMEDU	0.11	0.34	0.96	5.4	0.67
(5S,6S)-BMEDU	0.10	0.40	1.05	5.9	0.59
(5R,6R)-CMEDU	0.85	0.28	0.63	0.7	2.21
(5S,6R)-CMEDU	0.30	0.10	-	2.3	0.67

[a]Calculated using the Lagran program.
[b]Area under the curve of blood concentration *vs* time (0 → last sample).

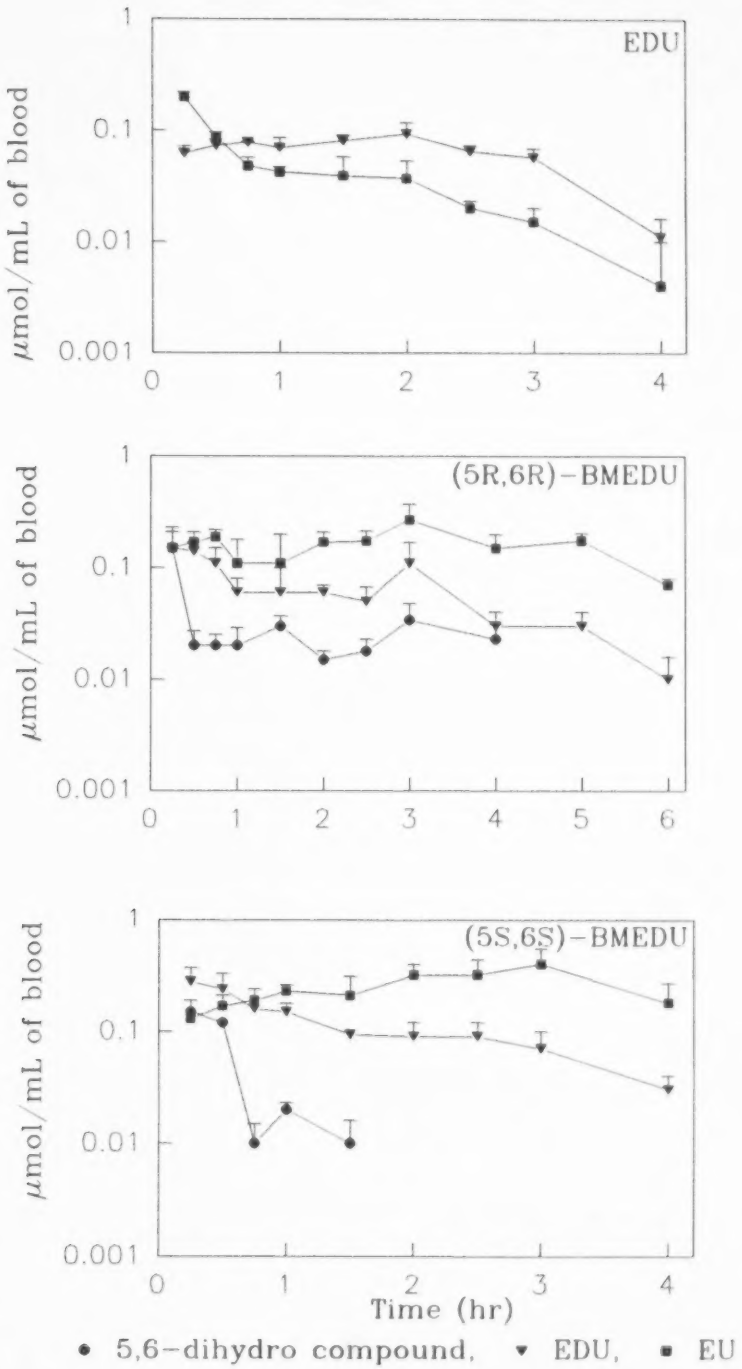


Figure 1.3.2. Concentration of 5,6-dihydro prodrug, EDU and EU in blood after iv injection of 0.7 mmol/kg of EDU, (5R,6R)-BMEDU and (5S,6S)-BMEDU into rats. Data are presented as the mean ± SD (n=3).

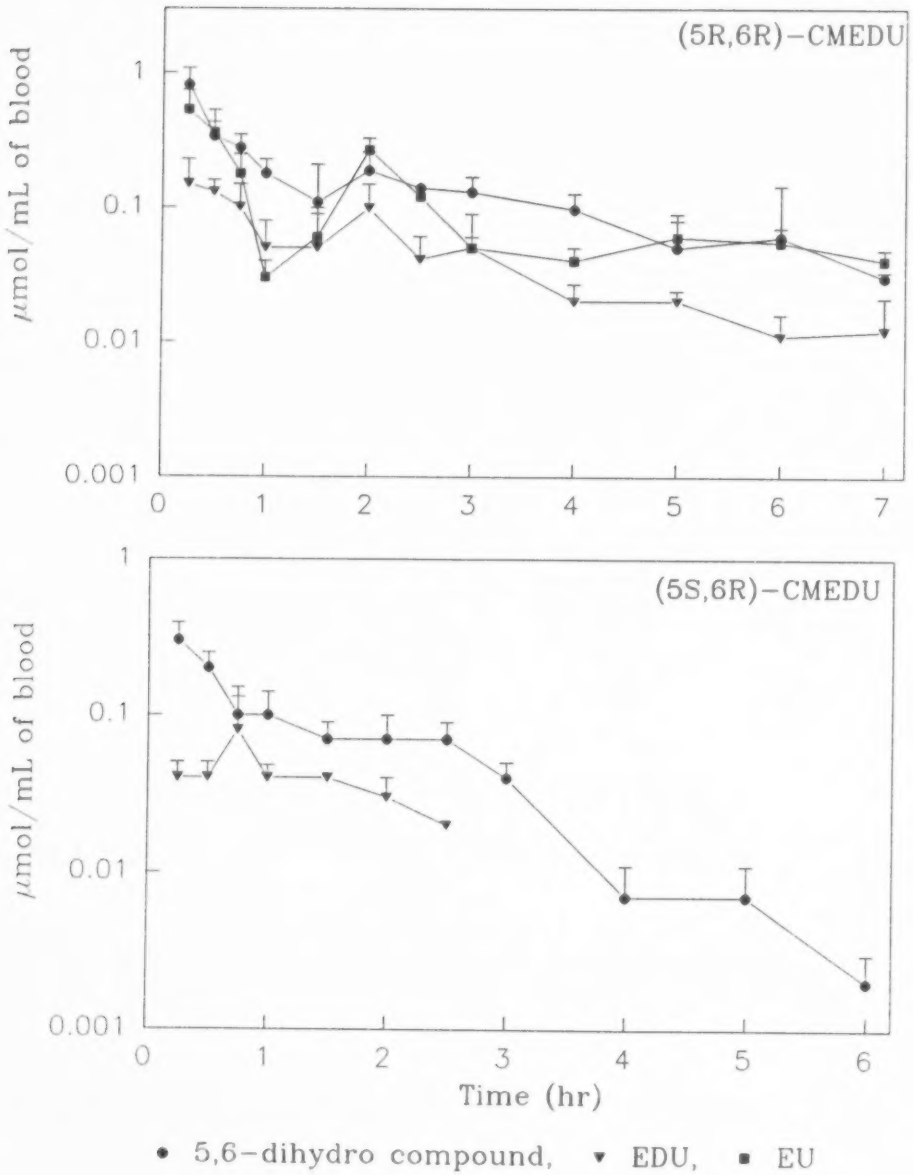


Figure 2.3.2. Concentration of 5,6-dihydro prodrug, EDU and EU in blood after iv injection of 0.7 mmol/kg of (5R,6R)-CMEDU and (5S,6R)-CMEDU into rats. Data are presented as the mean ± SD (n=3).

following injection of 0.7 mmol/kg of the test compound into the jugular vein of rats are summarized in Table 2.3.2 and figure 1.3.2 and 2.3.2.

(5R,6R)-CMEDU showed substantially longer half-life than EDU. However, the half-life of (5S,6S)-BMEDU was shorter than that of EDU and also this prodrug showed a higher total body clearance than that of EDU and other 5,6-dihydro prodrugs (Table 2.3.2). Although, (5S,6S)-BMEDU showed the lowest AUC relative to the other 5,6-dihydro prodrugs and EDU, it provided the highest concentration of EDU in blood samples. (5S,6R)-CMEDU provided the lowest concentration of EDU in blood. However, it showed a half-life similar to that of EDU. (5R,6R)-CMEDU showed the longest blood residence time and it was detected, in substantial concentration, even at 7 hr post injection. EU was detected and quantified as a secondary metabolite of EDU and all of the 5,6-dihydro prodrugs, except for (5S,6R)-CMEDU. 5-(1-Hydroxyethyl)-2'-deoxyuridine (HEDU) and 5-(1-hydroxyethyl)uracil (HEU) were detected, but not quantified, as metabolites of the 5,6-dihydro prodrugs.

3.3. Biodistribution study of [4-^{14}C]-labelled compounds

3.3.1. Tail vein injection of [4-^{14}C]-labelled EDU, (5R,6R)-BMEDU, (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU

Results from biodistribution studies for [4-^{14}C]-labelled EDU, (5S,6S)-BMEDU, (5R,6R)-BMEDU, BEEDU, VBEEDU and DVBEEDU following injection of a dose of 126 kBq (3.4 μCi), specific activity= 2 GBq (54mCi)/mmol, mixed with 0.2 mmol/kg of non-radioactive test compound

Table 3.3.3.1. Biodistribution of [4-^{14}C]-EDU at 3, 8, 18, 30, 60, 120 min,12 and 24 hr post injection of 126 kBq (3.4 μCi) into the tail vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min	120 min	12 hr	24 hr
Muscle	4.1E5 ± 3.0E4 5.4[a] 0.7[b]	1.5E5 ± 2.5E4 2.0 0.6	2.2E5 ± 2.8E4 2.9 1.0	3.4E5 ± 1.2E4 4.5 1.2	1.8E5 ± 2.3E5 2.4 1.3	6.2E4 ± 8.3E3 0.8 1.2	3.2E4 ± 6.4E3 0.4 2.9	1.1E4 ± 6.2E3 0.1 3.6
Bone	3.4E5 ± 3.9E4 4.5 0.6	1.2E5 ± 2.3E4 1.6 0.5	1.3E5 ± 2.4E4 1.7 0.6	1.4E5 ± 1.7E4 1.8 0.5	1.5E5 ± 1.2E4 2.0 1.1	5.7E4 ± 1.9E4 0.7 1.1	7.9E4 ± 1.0E4 1.0 7.2	2.2E4 ± 1.0E3 0.3 7.3
Brain	5.1E4 ± 6.4E3 0.7 0.08	3.3E4 ± 5.1E3 0.4 0.1	5.1E4 ± 2.4E3 0.7 0.2	8.9E4 ± 1.1E4 1.2 0.3	8.3E4 ± 5.7E3 1.1 0.6	3.8E4 ± 4.2E3 0.5 0.7	1.3E4 ± 1.3E3 0.2 1.2	3.4E3 ± 5.1E2 0.04 1.1
Lung	7.3E5 ± 1.1E5 9.6 1.2	2.1E5 ± 5.3E4 2.8 0.9	2.8E5 ± 4.9E4 3.7 1.3	3.6E5 ± 3.6E4 4.8 1.2	2.3E5 ± 3.1E4 3.0 1.6	9.2E4 ± 2.1E4 1.2 1.8	2.6E5 ± 2.0E5 3.4 23.6	2.5E4 ± 6.4E3 0.3 8.3
Fat	1.6E5 ± 4.5E4 2.1 0.3	5.8E4 ± 5.9E2 0.8 0.3	5.1E4 ± 1.2E4 0.7 0.2	1.3E5 ± 4.2E4 1.7 0.4	2.7E4 ± 5.4E3 0.4 0.2	1.4E4 ± 2.8E3 0.2 0.3	3.4E4 ± 1.8E4 0.4 3.1	7.6E3 ± 1.7E3 0.1 2.5
Blood	6.1E5 ± 1.1E5 8.1 1.0	2.3E5 ± 3.2E4 3.0 1.0	2.2E5 ± 4.2E4 2.9 1.0	2.9E5 ± 8.4E3 3.8 1.0	1.4E5 ± 1.5E4 1.8 1.0	5.1E4 ± 9.6E3 0.7 1.0	1.1E4 ± 3.4E3 0.1 1.0	3.0E3 ± 8.1E2 0.04 1.0
Liver	7.3E5 ± 5.3E4 9.7 1.2	2.7E5 ± 5.9E4 3.6 1.2	3.2E5 ± 2.5E4 4.2 1.5	3.9E5 ± 3.1E4 5.2 1.3	2.6E5 ± 3.2E4 3.4 1.8	1.5E5 ± 3.8E4 2.0 2.9	4.6E4 ± 1.1E4 0.1 4.2	1.4E4 ± 2.8E3 0.2 4.7
Large Intest.	ND[c]	ND	ND	3.2E5 ± 5.4E4 4.2 1.1	2.1E5 ± 3.9E4 2.8 1.5	2.0E5 ± 9.7E3 2.6 4.5	6.6E4 ± 1.8E5 0.9 6.0	2.3E5 ± 4.0E4 3.0 76.7
Small Intes.	ND	ND	ND	4.4E5 ± 9.4E4 5.8 1.5	2.7E5 ± 9.8E4 3.6 1.9	2.1E5 ± 4.2E4 2.8 2.2	3.0E5 ± 5.7E4 4.0 27.3	1.1E5 ± 3.0E4 1.5 36.7
Gall bladder[d]	ND	ND	ND	6.1E5 ± 1.6E5 8.1 2.1	7.7E5 ± 6.2E4 10.2 5.5	4.7E5 ± 1.4E5 6.2 9.2	1.3E5 ± 1.4E4 1.7 11.8	1.2E4 ± 2.8E3 0.3 4.0
Spleen	ND	ND	ND	3.1E5 ± 3.0E4 4.1 1.1	2.0E5 ± 2.6E4 2.6 1.4	9.3E4 ± 1.5E4 1.2 1.8	5.2E5 ± 4.6E3 0.7 4.7	2.0E4 ± 3.9E3 0.3 6.7
Kidney	ND	ND	ND	8.1E5 ± 1.9E5 10.7 2.8	2.8E5 ± 3.0E4 3.7 2.0	9.6E4 ± 1.6E4 1.3 1.9	2.6E4 ± 8.9E3 0.3 2.4	5.8E3 ± 1.5E3 0.08 1.9
Heart	ND	ND	ND	2.6E5 ± 1.6E4 3.4 0.9	1.7E5 ± 1.7E4 2.3 1.2	5.9E4 ± 1.1E4 0.8 1.2	1.7E4 ± 3.9E3 0.2 1.5	3.8E3 ± 1.1E3 0.05 1.3
Urine[e]	ND	ND	ND	8.9E6 ± 1.4E5 117.2 30.7	5.2E7 ± 1.9E7 80.2 44.5	2.6E6 ± 7.1E5 40.1 57.3	5.1E5 ± 1.0E5 6.7 46.4	1.3E5 ± 4.8E4 1.7 43.3

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg; [e]Activity as dpm/100 μL of urine, based on content of bladder at time of sacrifice

Table 4.3.3.1. Biodistribution of [4-^{14}C]-(5R,6R)-BMEDU at 3, 8, 18, 30, 60, 120 min, 12 and 24 hr post injection of 126 kBq (3.4 μCi) into the tail vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min	120 min	12 hr	24 hr
Muscle	4.1E5 ± 1.6E4 5.4[a] 0.8[b]	3.4E5 ± 9.4E4 4.5 0.6	2.9E5 ± 7.9E4 3.8 0.7	2.8E5 ± 1.2E5 3.7 0.7	1.6E5 ± 1.3E4 2.1 0.7	9.9E4 ± 8.9E3 1.3 0.5	1.8E4 ± 2.2E3 0.2 0.2	2.7E4 ± 3.7E3 0.4 0.4
Bone	3.9E5 ± 5.4E4 5.2 0.8	2.7E5 ± 5.8E4 3.6 0.5	3.3E5 ± 1.1E5 4.4 0.8	1.9E5 ± 7.2E4 2.5 0.5	1.3E5 ± 7.9E3 1.7 0.6	6.3E4 ± 1.4E4 0.8 0.3	4.5E4 ± 8.6E3 0.6 0.5	4.1E4 ± 1.6E4 0.5 0.6
Brain	5.0E4 ± 1.0E4 0.7 0.1	6.6E4 ± 1.3E4 0.9 0.1	7.9E4 ± 6.8E3 1.0 0.2	9.9E4 ± 4.9E3 1.3 0.3	8.9E4 ± 8.1E3 1.2 0.4	5.1E4 ± 1.3E4 0.7 0.3	5.5E3 ± 5.6E2 0.07 0.06	7.7E3 ± 2.9E3 0.1 0.1
Lung	8.8E5 ± 7.1E4 11.6 1.7	6.4E5 ± 1.3E5 8.5 1.1	4.8E5 ± 4.9E4 6.3 1.2	4.6E5 ± 1.1E5 6.1 1.2	2.1E5 ± 1.2E4 2.8 0.9	1.9E5 ± 6.7E4 2.5 1.0	3.7E4 ± 1.1E4 0.5 0.4	3.8E4 ± 9.1E3 0.5 0.6
Fat	7.6E4 ± 7.1E3 1.0 0.1	1.1E5 ± 1.5E5 1.5 0.2	6.6E4 ± 1.3E4 0.9 0.2	3.9E4 ± 1.1E4 0.5 0.1	1.9E4 ± 3.5E3 0.3 0.08	2.2E4 ± 5.9E3 0.3 0.1	3.7E3 ± 6.8E2 0.04 0.04	1.0E4 ± 4.6E3 0.1 0.2
Blood	5.2E5 ± 8.4E4 6.9 1.0	5.9E5 ± 6.3E4 7.8 1.0	4.0E5 ± 1.6E4 5.3 1.0	3.9E5 ± 5.4E4 5.2 1.0	2.3E5 ± 1.7E4 3.0 1.0	2.0E5 ± 6.0E4 2.6 1.0	8.9E4 ± 1.3E4 1.2 1.0	6.3E4 ± 2.3E4 0.8 1.0
Liver	8.6E5 ± 1.0E5 11.4 1.7	7.6E5 ± 6.9E4 10.0 1.3	4.8E5 ± 4.9E4 6.3 1.2	4.9E5 ± 1.1E5 6.5 1.3	2.5E5 ± 8.0E3 3.3 1.1	1.3E5 ± 5.1E4 1.7 0.7	3.2E4 ± 3.3E3 0.4 0.4	1.9E4 ± 6.2E3 0.3 0.3
Large Intest.	ND[c]	ND	ND	3.0E5 ± 4.5E4 4.0 0.8	3.3E5 ± 5.1E4 4.4 1.4	1.8E5 ± 3.9E4 2.4 0.9	4.8E5 ± 5.3E4 6.3 5.4	5.7E4 ± 1.1E4 0.8 0.9
Small Intes.	ND	ND	ND	3.3E5 ± 3.9E4 4.4 0.8	3.0E5 ± 6.6E4 4.0 1.3	2.3E5 ± 6.3E4 3.0 1.2	3.4E5 ± 1.4E5 4.5 3.8	2.5E5 ± 1.6E5 3.3 4.0
Gall bladder[d]	ND	ND	ND	5.8E5 ± 5.1E4 7.7 1.5	3.9E5 ± 1.1E5 5.2 1.7	4.7E5 ± 2.2E5 6.2 2.4	2.4E4 ± 9.7E3 0.3 0.3	1.1E4 ± 3.9E3 0.1 0.2
Spleen	ND	ND	ND	3.4E5 ± 1.0E5 4.5 0.9	2.2E5 ± 8.9E3 2.9 1.0	1.4E5 ± 2.6E4 1.9 0.7	4.0E4 ± 5.3E3 0.5 0.4	3.5E4 ± 9.2E3 0.5 0.6
Kidney	ND	ND	ND	7.1E5 ± 7.9E4 9.4 1.8	2.8E5 ± 3.8E3 3.7 1.2	1.4E5 ± 1.9E4 1.9 0.7	2.6E4 ± 2.7E3 0.3 0.3	1.6E4 ± 4.5E3 0.2 0.3
Heart	ND	ND	ND	3.3E5 ± 1.2E5 4.4 0.8	1.9E5 ± 1.5E4 2.5 0.8	1.1E5 ± 2.1E4 1.5 0.6	2.6E4 ± 1.6E3 0.3 0.3	2.0E4 ± 5.2E3 0.3 0.3
Urine[e]	ND	ND	ND	3.7E6 ± 5.5E5 48.9 9.5	1.0E6 ± 2.3E5 13.2 4.3	8.4E5 ± 1.9E5 11.1 4.2	1.1E5 ± 2.8E4 1.5 1.2	6.1E4 ± 2.6E4 0.8 1.0

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg; [e]Activity as dpm/100 μL of urine, based on content of bladder at time of sacrifice

Table 5.3.3.1. Biodistribution of [4-^{14}C]-(5S,6S)-BMEDU at 3, 8, 18, 30, 60, 120 min, 12 and 24 hr post injection of 126 kBq (3.4 μCi) into the tail vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min	120 min	12 hrs	24 hrs
Muscle	3.3E5 ± 4.3E4 4.4[a] 0.5[b]	4.3E5 ± 4.2E4 5.7 0.9	2.7E5 ± 5.8E4 3.6 0.7	3.3E5 ± 8.7E4 4.4 1.1	2.4E5 ± 1.4E5 3.2 1.7	1.4E5 ± 4.4E4 1.9 0.6	9.9E3 ± 3.5E3 0.1 0.2	5.0E4 ± 3.8E4 0.7 1.5
Bone	3.8E5 ± 8.0E4 5.0 0.6	4.1E5 ± 6.8E4 5.4 0.9	1.6E5 ± 2.7E4 2.1 0.4	1.1E5 ± 1.1E4 1.5 0.4	7.7E4 ± 3.2E4 1.0 0.6	1.4E5 ± 3.4E4 1.9 0.6	2.3E4 ± 7.3E3 0.3 0.6	3.1E4 ± 1.4E4 0.4 0.9
Brain	5.4E4 ± 1.5E4 0.7 0.09	6.2E4 ± 6.2E3 0.8 0.1	6.2E4 ± 1.0E4 0.8 0.2	5.8E4 ± 7.7E3 0.8 0.2	5.7E4 ± 1.8E4 0.8 0.4	7.5E4 ± 2.3E4 1.0 0.3	4.3E3 ± 6.6E2 0.05 0.1	3.7E3 ± 6.4E2 0.04 0.1
Lung	5.6E5 ± 9.3E4 7.4 0.9	7.9E5 ± 2.9E4 10.4 0.6	6.4E5 ± 7.5E4 8.5 1.7	3.4E5 ± 1.0E5 4.5 1.2	1.8E5 ± 3.4E4 2.4 1.3	1.9E5 ± 5.3E4 2.5 0.8	3.1E4 ± 9.9E3 0.4 0.8	5.1E4 ± 1.2E4 0.7 0.2
Fat	7.5E4 ± 1.6E4 1.0 0.1	6.6E4 ± 7.3E3 0.9 0.1	5.4E4 ± 8.1E3 0.7 0.1	6.1E4 ± 8.1E3 0.8 0.2	5.0E4 ± 1.4E4 0.7 0.4	4.3E4 ± 9.6E3 0.6 0.4	1.2E4 ± 6.9E3 0.2 0.3	2.3E4 ± 6.4E3 0.3 0.7
Blood	6.1E5 ± 2.1E4 8.1 1.0	4.8E5 ± 1.8E4 6.3 1.0	3.8E5 ± 3.1E4 5.0 1.0	2.9E5 ± 4.5E4 3.8 1.0	1.9E5 ± 6.2E4 2.5 1.0	2.5E5 ± 7.3E4 3.3 1.0	4.1E4 ± 7.1E3 0.5 1.0	3.4E4 ± 1.5E4 0.4 1.0
Liver	7.6E5 ± 1.1E5 10.1 1.2	7.8E5 ± 6.7E4 10.3 1.6	4.0E5 ± 7.3E4 5.3 1.0	3.1E5 ± 7.6E4 4.1 1.1	1.6E5 ± 2.9E4 2.1 1.1	2.0E5 ± 7.7E4 2.6 0.8	1.8E4 ± 2.5E3 0.2 0.4	2.7E4 ± 1.1E4 0.4 0.8
Large Intest.	ND[c]	ND	ND	1.6E5 ± 4.1E4 2.1 0.6	2.5E5 ± 9.2E4 3.3 1.8	2.3E5 ± 3.6E4 3.0 0.9	3.1E5 ± 4.1E4 4.1 7.6	1.1E5 ± 3.2E4 1.5 3.2
Small Intest.	ND	ND	ND	2.2E5 ± 5.9E4 2.9 0.8	1.3E5 ± 3.5E4 1.7 0.9	4.6E5 ± 1.8E5 6.1 1.8	2.0E5 ± 6.9E4 2.6 4.9	2.5E5 ± 1.2E5 3.3 7.3
Gall bladder[d]	ND	ND	ND	1.1E5 ± 2.7E4 1.5 0.4	9.6E4 ± 5.6E4 1.3 0.7	1.5E5 ± 5.7E4 2.0 0.6	4.2E4 ± 1.4E4 0.5 1.0	1.3E4 ± 3.1E3 0.2 0.4
Spleen	ND	ND	ND	3.0E5 ± 7.1E4 4.0 1.0	1.5E5 ± 2.7E4 2.0 1.1	2.6E5 ± 3.3E4 3.4 1.0	3.3E4 ± 5.6E2 0.4 0.8	3.1E4 ± 6.9E3 0.4 0.9
Kidney	ND	ND	ND	4.6E5 ± 1.1E5 6.1 1.6	2.1E5 ± 4.2E4 2.8 1.5	2.5E5 ± 7.4E4 3.3 1.0	1.3E4 ± 1.6E3 0.2 0.3	1.3E4 ± 3.0E3 0.2 0.4
Heart	ND	ND	ND	3.0E5 ± 8.3E4 4.0 1.0	1.5E5 ± 2.9E4 2.0 1.1	1.8E5 ± 5.6E4 2.4 0.7	1.6E4 ± 2.6E3 0.2 0.4	2.4E4 ± 5.5E3 0.3 0.7
Urine[e]	ND	ND	ND	8.4E5 ± 2.7E5 11.1 2.9	9.1E5 ± 2.8E5 12.0 6.5	1.9E6 ± 2.1E6 25.1 7.6	3.6E5 ± 2.9E5 4.8 8.8	1.6E4 ± 1.1E4 0.2 0.5

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg; [e]Activity as dpm/100 μL of urine, based on content of bladder at time of sacrifice

Table 6.3.3.1. Biodistribution of [4-^{14}C]-BEEDU at 3, 8, 18, 30, 60 and 120 min post injection of 126 kBq (3.4 μCi) into the tail vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min	120 min
Muscle	5.1E5 ± 2.2E4 6.7[a] 1.1[b]	7.3E5 ± 2.6E5 9.7 1.6	7.2E5 ± 1.2E5 9.5 1.8	1.9E5 ± 3.2E4 2.5 0.8	8.5E4 ± 1.7E4 1.1 0.7	7.3E4 ± 1.3E4 1.0 0.7
Bone	1.7E5 ± 1.0E4 2.3 0.4	4.7E5 ± 2.2E5 6.2 1.1	1.6E5 ± 2.1E4 2.1 0.4	1.5E5 ± 3.2E4 2.0 0.6	6.1E4 ± 7.6E3 0.8 0.5	7.7E4 ± 1.7E4 1.0 0.8
Brain	4.6E4 ± 1.7E3 0.6 0.1	1.0E5 ± 7.7E3 1.3 0.2	8.5E4 ± 1.5E4 1.1 0.2	1.1E5 ± 1.2E4 1.5 0.5	5.2E4 ± 8.4E3 0.7 0.4	4.7E4 ± 6.9E3 0.6 0.5
Lung	1.2E6 ± 1.8E5 15.9 2.6	1.1E6 ± 3.4E5 14.6 2.5	1.5E6 ± 3.0E5 19.9 3.8	3.2E5 ± 8.8E4 4.2 1.4	1.1E5 ± 1.8E4 1.5 0.8	1.4E5 ± 1.5E4 1.8 1.4
Fat	7.3E4 ± 1.4E4 1.0 0.2	6.5E4 ± 1.0E4 0.9 0.1	1.3E5 ± 1.8E4 1.7 0.3	7.1E4 ± 3.1E3 0.9 0.3	2.0E4 ± 5.4E3 0.3 0.2	1.9E4 ± 2.3E3 0.3 0.2
Blood	4.6E5 ± 1.3E4 6.1 1.0	4.4E5 ± 3.3E4 5.8 1.0	3.9E5 ± 5.3E4 5.2 1.0	2.3E5 ± 4.2E4 3.0 1.0	1.3E5 ± 3.4E4 1.7 1.0	1.0E5 ± 1.3E4 1.3 1.0
Liver	1.2E6 ± 1.2E5 15.9 2.6	7.5E5 ± 2.1E5 10.0 1.7	4.1E5 ± 1.0E5 5.4 1.1	3.1E5 ± 4.5E4 4.1 1.3	1.5E5 ± 2.2E4 2.0 1.2	1.3E5 ± 1.2E4 1.7 1.3
Large Intest.	ND[c]	ND	ND	2.1E5 ± 6.1E4 2.8 0.9	2.1E5 ± 3.5E4 2.8 1.6	3.7E5 ± 1.8E4 4.9 3.7
Small Intest.	ND	ND	ND	3.5E5 ± 1.1E5 4.6 1.5	1.7E5 ± 1.6E4 2.2 1.3	3.2E5 ± 5.8E4 4.2 3.2
Gall bladder[d]	ND	ND	ND	1.8E5 ± 4.0E4 2.4 0.8	2.3E5 ± 5.6E4 3.0 1.8	1.4E5 ± 2.0E4 1.9 1.4
Spleen	ND	ND	ND	2.3E5 ± 4.8E4 3.0 1.0	1.3E5 ± 2.1E4 1.7 1.0	9.7E4 ± 5.1E4 1.3 1.0
Kidney	ND	ND	ND	3.8E5 ± 6.1E4 5.0 1.7	1.5E5 ± 1.9E4 2.0 1.2	1.4E5 ± 1.6E4 1.9 1.4
Heart	ND	ND	ND	2.3E5 ± 4.7E4 3.0 1.0	1.1E5 ± 1.8E4 1.5 1.0	8.8E4 ± 1.0E4 1.2 0.9
Urine[e]	ND	ND	ND	2.1E6 ± 2.6E5 32.4 10.8	1.7E6 ± 2.9E5 26.2 15.4	7.2E5 ± 1.3E5 11.1 8.5

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg; [e]Activity as dpm/100 μL of urine, based on content of bladder at time of sacrifice

Table 7.3.3.1. Biodistribution of [4-^{14}C]-VBEEDU at 3, 8, 18, 30, 60, and 120 min post injection of 126 kBq (3.4 μCi) into the tail vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min	120 min
Muscle	2.9E5 ± 1.3E5 3.8[a] 1.0[b]	3.1E5 ± 6.3E4 4.1 1.2	1.8E5 ± 4.1E4 2.4 0.7	1.8E5 ± 4.3E3 2.3 1.0	1.7E5 ± 2.9E4 2.2 0.9	5.1E4 ± 2.8E3 0.7 0.7
Bone	1.3E5 ± 2.6E4 1.7 0.4	2.0E5 ± 1.9E4 2.6 0.8	1.5E5 ± 2.5E4 2.0 0.6	1.6E5 ± 6.2E4 2.1 0.9	1.1E5 ± 1.7E4 1.5 0.6	7.1E4 ± 2.1E4 0.9 1.0
Brain	1.7E5 ± 3.2E4 2.2 0.6	7.6E4 ± 1.0E4 1.0 0.3	1.3E5 ± 1.0E4 1.7 0.5	8.4E4 ± 6.2E3 1.1 0.5	7.0E4 ± 3.6E3 0.9 0.4	3.3E4 ± 3.4E3 0.4 0.5
Lung	8.8E5 ± 6.0E4 11.6 3.0	4.1E5 ± 5.9E4 5.4 1.6	3.5E5 ± 1.3E5 4.6 1.3	4.3E5 ± 1.9E5 5.7 2.4	1.5E5 ± 2.1E4 2.0 0.8	7.1E4 ± 5.8E3 0.9 1.0
Fat	7.5E4 ± 1.0E4 1.0 0.3	6.2E4 ± 1.3E4 0.8 0.2	3.5E4 ± 2.5E3 0.5 0.1	4.8E4 ± 4.1E3 0.6 0.3	3.4E4 ± 5.8E3 0.4 0.2	1.9E4 ± 2.7E3 0.3 0.3
Blood	2.9E5 ± 4.3E3 3.8 1.0	2.5E5 ± 2.7E4 3.3 1.0	2.7E5 ± 1.3E4 3.6 1.0	1.8E5 ± 5.1E4 2.4 1.0	1.8E5 ± 7.8E3 2.4 1.0	6.9E4 ± 7.4E3 0.9 1.0
Liver	4.1E5 ± 7.0E4 5.4 1.4	5.3E5 ± 8.0E4 7.0 2.1	5.5E5 ± 7.2E4 7.3 2.0	3.5E5 ± 1.0E4 4.6 1.9	2.9E5 ± 3.5E4 3.8 1.6	1.2E5 ± 3.6E3 1.6 1.7
Large Intest.	ND[c]	ND	1.3E5 ± 2.3E4 1.7 0.5	1.8E5 ± 3.1E4 2.4 1.0	1.6E5 ± 1.8E4 2.1 0.9	1.7E5 ± 7.5E4 2.2 2.5
Small Intest.	ND	ND	1.4E5 ± 4.3E4 1.9 0.5	5.9E5 ± 5.4E4 7.8 3.3	5.7E5 ± 1.5E5 7.5 3.2	1.5E5 ± 1.9E4 2.0 2.2
Gall bladder[d]	ND	ND	8.7E4 ± 1.6E4 1.2 0.3	4.5E5 ± 3.3E5 6.0 2.5	5.2E5 ± 3.1E5 6.9 2.9	5.6E4 ± 4.4E3 0.7 0.8
Spleen	ND	ND	2.1E5 ± 7.7E4 2.8 0.8	2.9E5 ± 2.4E4 3.8 1.6	1.9E5 ± 3.4E4 2.5 1.1	1.4E5 ± 5.4E4 1.9 2.0
Kidney	ND	ND	3.6E5 ± 8.9E4 4.8 1.3	3.3E5 ± 2.3E4 4.4 1.8	2.4E5 ± 1.5E4 3.2 1.3	9.9E4 ± 8.9E3 1.3 1.4
Heart	ND	ND	3.0E5 ± 6.2E4 4.0 1.1	1.8E5 ± 5.8E3 2.4 1.0	2.0E5 ± 7.0E4 2.6 1.1	5.4E4 ± 4.9E3 0.7 0.8
Urine[e]	ND	ND	4.9E5 ± 2.3E5 6.5 1.8	5.0E6 ± 1.4E5 66.1 27.8	7.7E6 ± 5.2E5 101.8 42.8	4.1E6 ± 6.1E5 54.2 59.4

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg; [e]Activity as dpm/100 μL of urine, based on content of bladder at time of sacrifice

Table 8.3.3.1. Biodistribution of [4-^{14}C]-DVBEEDU at 3, 8, 18, 30, 60 and 120 min post injection of 126 kBq (3.4 μCi) into the tail vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min	120 min
Muscle	4.5E5 ± 8.1E4 5.9[a] 0.9[b]	3.8E5 ± 1.6E4 5.0 1.3	4.0E5 ± 1.6E5 5.3 1.1	3.3E5 ± 2.0E4 4.4 1.0	1.8E5 ± 4.2E4 2.4 1.0	5.7E4 ± 1.6E4 0.8 0.7
Bone	1.7E5 ± 6.1E4 2.3 0.4	1.8E5 ± 2.2E3 2.4 0.6	1.8E5 ± 4.4E4 2.4 0.5	1.8E5 ± 2.1E4 2.4 0.5	1.4E5 ± 5.6E4 1.8 0.8	5.8E4 ± 1.3E4 0.8 0.7
Brain	1.9E5 ± 5.6E3 2.5 0.4	1.1E5 ± 1.3E4 1.5 0.4	1.3E5 ± 3.0E4 1.7 0.4	1.3E5 ± 1.8E4 1.7 0.4	9.0E4 ± 1.8E4 1.2 0.5	4.4E4 ± 5.1E3 0.6 0.6
Lung	1.5E6 ± 2.5E5 19.8 3.1	6.4E5 ± 7.1E4 8.5 2.1	5.1E5 ± 1.9E5 6.7 1.4	6.0E5 ± 9.1E4 7.9 1.8	2.4E5 ± 6.8E4 3.2 1.3	7.7E4 ± 1.8E4 1.0 1.0
Fat	6.8E4 ± 1.2E4 0.9 0.1	8.9E4 ± 2.9E4 1.2 0.3	9.0E4 ± 3.9E3 1.2 0.2	1.7E5 ± 9.4E3 2.2 0.5	8.4E4 ± 3.2E4 1.1 0.5	3.9E4 ± 1.0E3 0.5 0.5
Blood	4.8E5 ± 6.2E4 6.3 1.0	3.0E5 ± 2.5E4 4.0 1.0	3.7E5 ± 4.0E4 4.9 1.0	3.3E5 ± 2.8E4 4.4 1.0	1.8E5 ± 1.9E4 2.4 1.0	7.8E4 ± 1.5E4 1.0 1.0
Liver	1.4E6 ± 2.6E5 18.5 2.9	7.3E5 ± 1.1E5 9.6 2.4	7.6E5 ± 8.1E4 10.1 2.1	5.6E5 ± 7.7E4 7.4 1.7	3.1E5 ± 1.9E4 4.1 1.7	1.3E5 ± 7.7E3 1.7 1.7
Large Intest.	ND[c]	ND	ND	1.3E5 ± 1.3E4 1.7 0.4	3.5E5 ± 6.1E3 4.6 1.9	4.1E5 ± 7.6E4 5.4 5.3
Small Intest.	ND	ND	ND	2.8E5 ± 1.5E4 3.7 0.8	2.3E5 ± 4.3E4 3.0 1.3	2.1E5 ± 1.7E4 2.8 2.7
Gall bladder[d]	ND	ND	ND	5.8E4 ± 2.5E4 0.8 0.2	1.1E5 ± 2.2E4 1.5 0.6	3.0E4 ± 1.3E4 0.4 0.4
Spleen	ND	ND	ND	2.7E5 ± 3.3E4 3.6 0.8	2.4E5 ± 4.2E4 3.2 1.3	1.0E5 ± 2.3E4 1.3 1.3
Kidney	ND	ND	ND	6.0E5 ± 1.3E5 7.9 1.8	2.5E5 ± 3.7E4 3.3 1.4	8.7E4 ± 2.0E4 1.2 1.1
Heart	ND	ND	ND	3.4E5 ± 1.3E4 4.5 1.0	1.7E5 ± 3.6E4 2.2 0.9	5.5E4 ± 1.6E4 0.3 0.7
Urine[e]	ND	ND	ND	3.5E6 ± 5.0E5 46.3 10.6	8.9E6 ± 9.9E5 117.7 49.4	5.9E6 ± 2.5E6 78.0 75.6

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg; [e]Activity as dpm/100 μL of urine, based on content of bladder at time of sacrifice

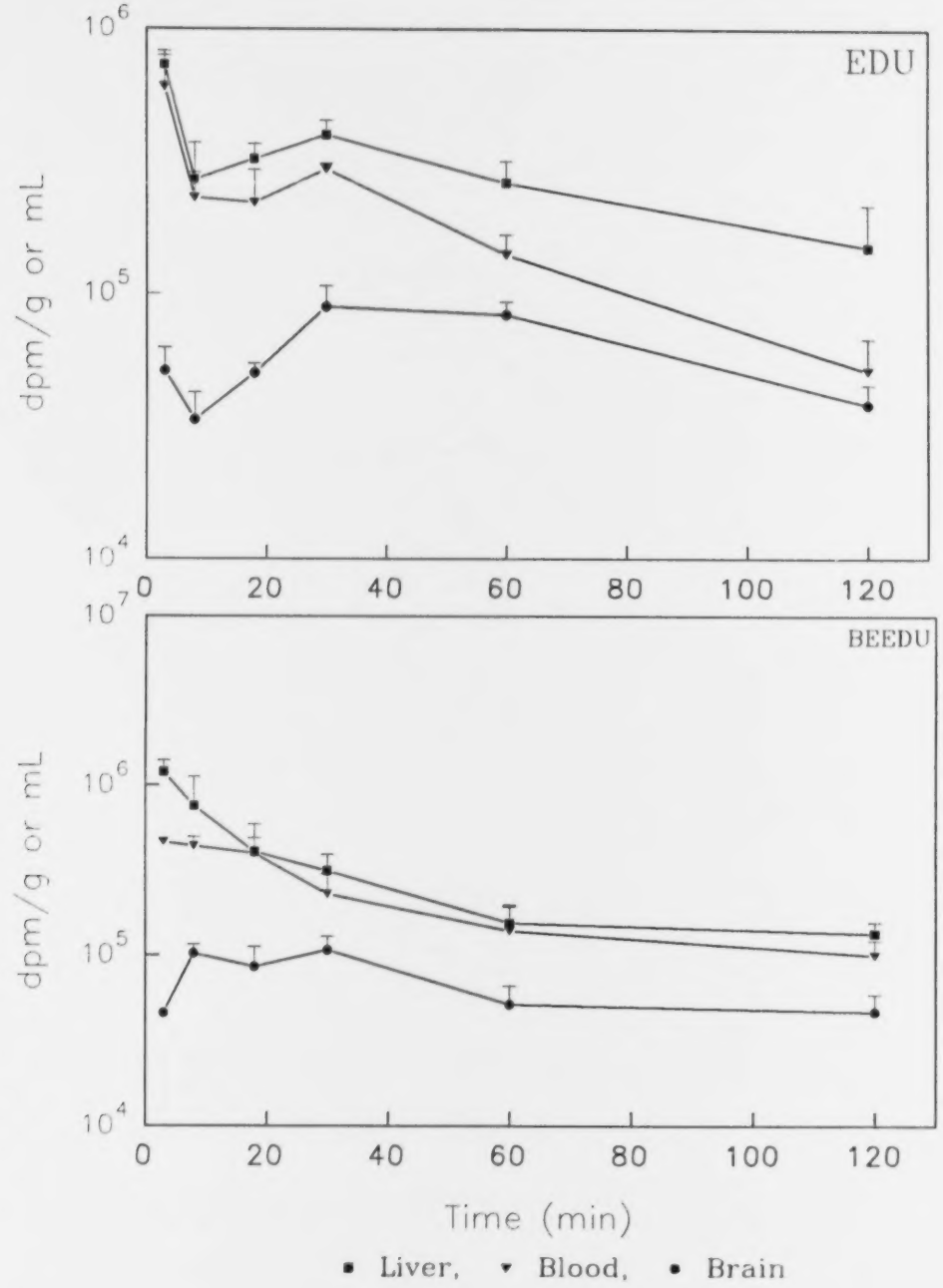


Figure 3.3.3.1. Distribution of radioactivity in brain, blood and liver samples as a function of time after injection of [4-^{14}C]-EDU or [4-^{14}C]-BEEDU. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

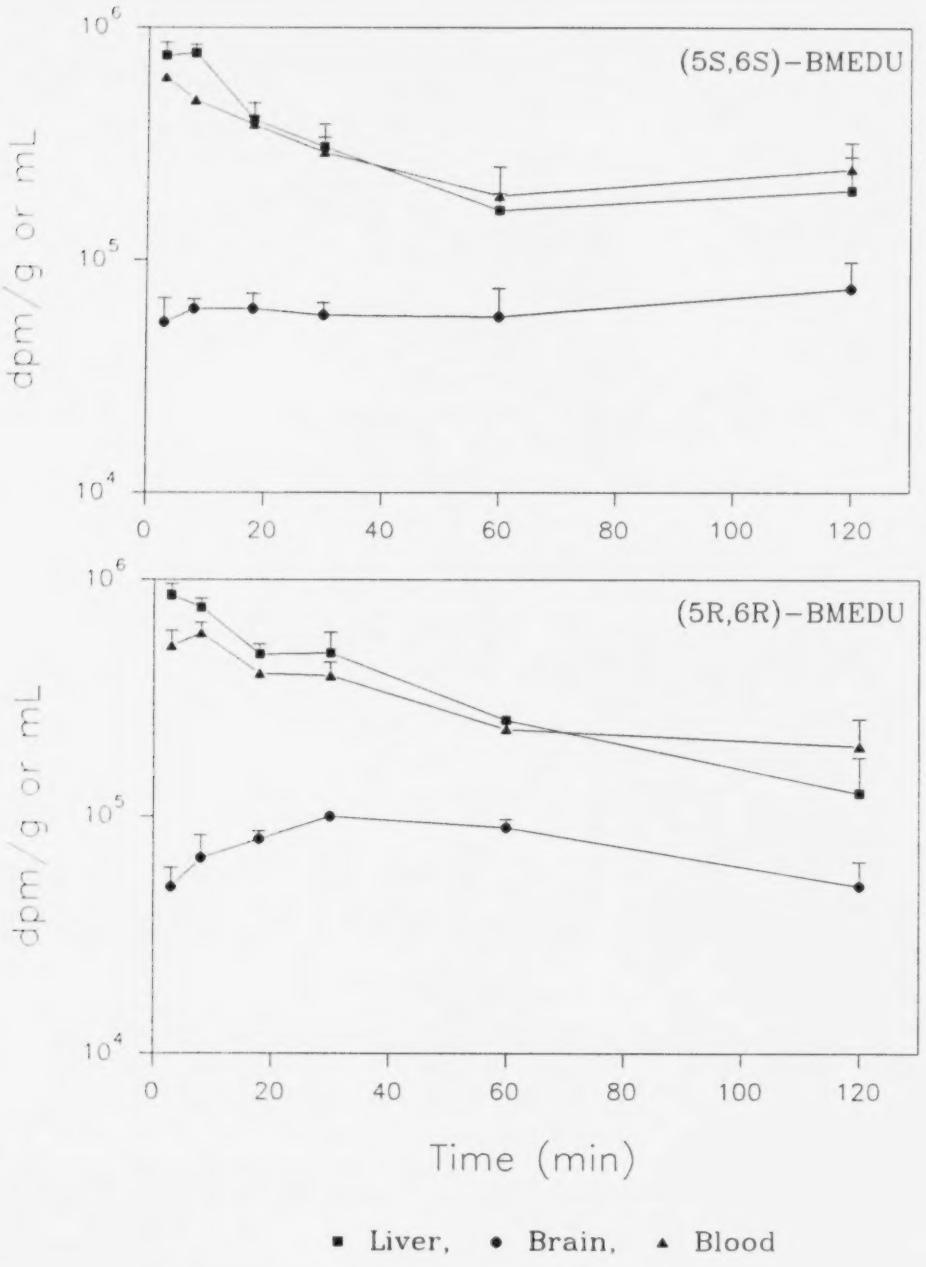


Figure 4.3.3.1. Distribution of radioactivity in brain, blood and liver samples as a function of time after injection of [4-^{14}C]-(5S,6S)-BMEDU or [4-^{14}C]-(5R,6R)-BMEDU. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

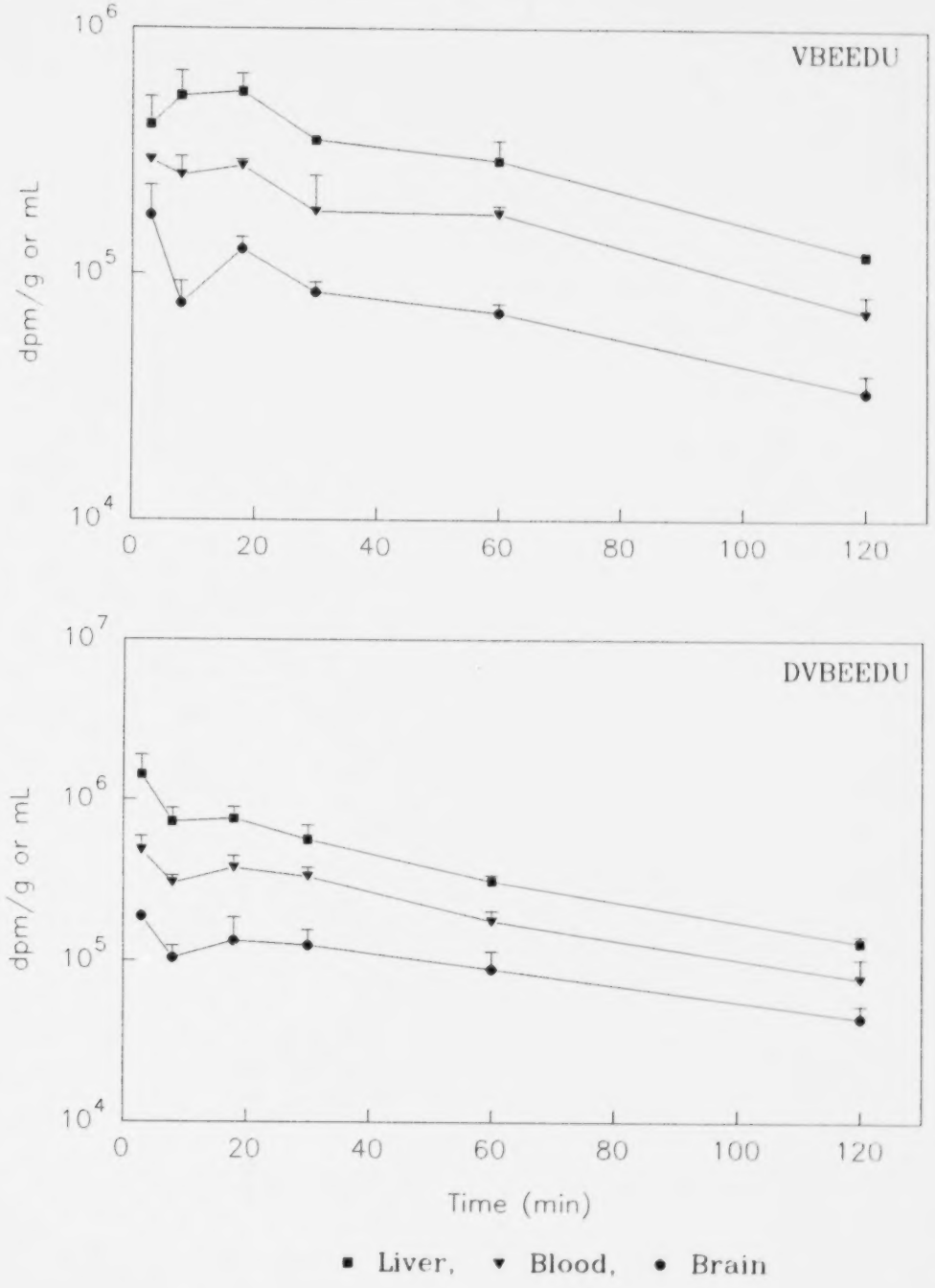


Figure 5.3.3.1. Distribution of radioactivity in brain, blood and liver samples as a function of time after injection of [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

into the tail vein of male Balb-C mice are tabulated in Tables 3.3.3.1 to 8.3.3.1. Distribution of radioactivity in brain, blood and liver samples are also shown in figures 4.3.3.1 to 6.3.3.1.

A rapid clearance of radioactivity was observed for blood samples taken following injection of [4-^{14}C]-EDU. This rapid clearance of radioactivity was observed in liver and almost all other tissue samples. Tissue samples taken 8 min post injection of [4-^{14}C]-EDU showed significantly lower radioactivity levels than those taken 3 min post injection. Urine samples taken between 30-120 min post injection of [4-^{14}C]-EDU contained a substantial amount of radioactivity. However, no significant amount of radioactivity was found in tissue samples taken at 12 hr and 24 hr post injection of [4-^{14}C]-EDU. Liver samples showed higher levels of radioactivity than those of blood samples. Total amount of radioactivity found per 100 mg of gall bladder was more than 8% and 10% of injected dose at 30 and 60 min post injection of [4-^{14}C]-EDU. In contrast to the high radioactivity levels in lung samples, brain samples taken following injection of [4-^{14}C]-EDU did not show more than 1.2% of the injected dose at any sampling time interval. The brain samples taken at 30 min post injection showed the highest radioactivity levels.

The overall distribution of radioactivity in tissues following injection of [4-^{14}C]-labelled (5R,6R)-BMEDU and (5S,6S)-BMEDU was different from each other and from that of EDU (Figure 3.3.3.1 and 4.3.3.1, Tables 3.3.3.1 to 5.3.3.1). Liver samples taken after injection for [4-^{14}C]-labelled BMEDUs showed higher radioactivity levels than those of [4-^{14}C]-EDU. Clearance of radioactivity from tissues taken after injection of [4-^{14}C]-labelled BMEDUs was slower than that of [4-^{14}C]-EDU.

Liver samples taken at < 60 min post injection of [4-^{14}C]-(5S,6S)-BMEDU showed higher radioactivity levels than those of the corresponding blood samples. Radioactivity levels in tissue samples, including blood, brain and liver, taken at > 30 min post injection of [4-^{14}C]-(5S,6S)-BMEDU showed a plateau. Brain samples taken between 3-120 min post injection of [4-^{14}C]-(5S,6S)-BMEDU showed similar radioactivity levels to those of [4-^{14}C]-EDU. Distribution of radioactivity in brain samples taken following injection of [4-^{14}C]-labelled (5R,6R)-BMEDU or (5S,6S)-BMEDU was not significantly different ($P > 0.05$) than those for [4-^{14}C]-EDU. Urine and gall bladder samples taken at > 18 min post injection of [4-^{14}C]-(5S,6S)-BMEDU showed significantly lower radioactivity levels than that of [4-^{14}C]-EDU. However, the overall radioactivity levels in lung samples taken after injection of [4-^{14}C]-(5S,6S)-BMEDU were higher than those of for [4-^{14}C]-EDU.

Liver samples taken after injection of [4-^{14}C]-(5R,6R)-BMEDU showed a higher uptake of radioactivity compared to those of [4-^{14}C]EDU and [4-^{14}C]-(5S,6S)-BMEDU. Liver samples taken at < 120 min post injection of [4-^{14}C]-(5R,6R)-BMEDU also showed substantially higher radioactivity levels than those of the corresponding blood samples. Brain samples taken following injection of [4-^{14}C]-(5R,6R)-BMEDU showed a C_{max} at 30 min post injection. Radioactivity levels in gall bladder samples taken following injection of [4-^{14}C]-(5R,6R)-BMEDU were significantly higher than those of [4-^{14}C]-(5S,6S)-BMEDU, whereas urine samples showed lower radioactivity levels than those after injection of [4-^{14}C]-EDU. Lung samples taken after injection of [4-^{14}C]-(5R,6R)-BMEDU also showed high levels of radioactivity.

Very high levels of radioactivity were found in muscle, lung and liver samples following injection of [4-^{14}C]-BEEDU (Table 6.3.3.1). Liver samples, especially those taken shortly after injection of [4-^{14}C]-BEEDU, also showed higher radioactivity levels than those of corresponding blood samples. The overall distribution of radioactivity in brain samples taken following injection of [4-^{14}C]-BEEDU was different from those after [4-^{14}C]-EDU and [4-^{14}C]-labelled BMEDUs. Brain samples taken after injection of [4-^{14}C]-BEEDU showed significantly higher ($P < 0.05$) levels of radioactivity than those of [4-^{14}C]-EDU. Compared to [4-^{14}C]-EDU, urine samples taken after injection of [4-^{14}C]-BEEDU showed lower radioactivity levels.

Liver samples taken following injection of [4-^{14}C]-VBEEDU showed lower radioactivity levels than those for [4-^{14}C]-BEEDU, but were higher than those for [4-^{14}C]-EDU. However, liver samples still had higher radioactivity levels than those of the corresponding blood samples. Brain samples taken following injection of [4-^{14}C]-VBEEDU showed significantly higher ($P < 0.001$) radioactivity levels than those after [4-^{14}C]-EDU. The highest radioactivity level among the samples taken between 3-120 min post injection of [4-^{14}C]-VBEEDU was observed in brain samples taken at 3 min post injection (2.2% of injected dose). Radioactivity levels in lung samples taken after injection of [4-^{14}C]-VBEEDU were also elevated.

Liver samples taken following injection of [4-^{14}C]-DVBEEDU contained very high levels of radioactivity. The level of radioactivity in liver samples was significantly higher than those of blood samples. However, lung samples also showed very high radioactivity levels after injection of [4-^{14}C]-DVBEEDU. Brain samples taken following injection of [4-^{14}C]-DVBEEDU showed significantly higher ($P <0.001$) radioactivity levels compared to those after [4-^{14}C]-EDU. However, brain samples taken after

injection of [4-^{14}C]-DVBEEDU showed similar radioactivity levels to those for [4-^{14}C]-VBEEDU.

3.3.2. Jugular vein injection of [4-^{14}C]-labelled BEEDU, VBEEDU and DVBEEDU

Jugular injection of [4-^{14}C]-BEEDU into male Balb-C mice did not provide significantly higher ($P > 0.05$) radioactivity levels in brain samples compared to those for tail vein injection. Lung samples taken after jugular injection of [4-^{14}C]-BEEDU showed slightly lower radioactivity levels (Tables 6.3.3.1 and 9.3.3.2).

Liver samples taken following jugular injection of [4-^{14}C]-VBEEDU did not show substantially different radioactivity levels than those following tail vein injection (Tables 7.3.3.1 and 10.3.3.2). However, much higher radioactivity levels were observed in blood and lung samples taken after jugular injection of [4-^{14}C]-VBEEDU. Injection of [4-^{14}C]-VBEEDU into the jugular vein provided significantly higher ($p < 0.001$) radioactivity levels in brain samples, with the highest radioactivity level (3.6% of the injected dose) in samples taken 3 min post injection, compared to those after tail vein injection.

Jugular vein injection of [4-^{14}C]-DVBEEDU also provided significantly higher radioactivity ($P < 0.001$) levels in brain samples compared to those for the tail vein injection route. The highest radioactivity level in brain samples taken following jugular vein injection of [4-^{14}C]-DVBEEDU was observed in samples taken 3 min post injection (Table 11.3.3.2). Liver samples taken shortly after jugular injection of [4-^{14}C]-DVBEEDU showed lower radioactivity levels than those for the tail vein injection route. In contrast to much higher radioactivity levels in lung

Table 9.3.3.2. Biodistribution of [4-^{14}C]-BEEDU at 3, 8, 18, 30 and 60 min post injection of 126 kBq (3.4 μCi) into the jugular vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min
Muscle	2.7E5 ± 1.0E5 3.6[a] 0.5[b]	6.7E5 ± 1.0E5 8.9 1.8	4.2E5 ± 3.2E4 5.6 1.0	3.5E5 ± 3.3E4 4.6 0.7	3.1E5 ± 2.5E4 4.1 0.8
Bone	1.9E5 ± 4.5E5 2.5 0.4	2.1E5 ± 3.8E4 2.8 0.6	1.6E5 ± 2.5E4 2.1 0.4	2.3E5 ± 5.6E4 3.0 0.5	1.6E5 ± 2.6E4 2.1 0.4
Brain	5.9E4 ± 1.4E4 0.8 0.1	4.9E4 ± 2.1E4 0.6 0.1	7.8E4 ± 1.1E4 1.0 0.2	7.3E4 ± 1.2E4 1.0 0.1	8.6E4 ± 1.0E4 1.1 0.2
Lung	9.2E5 ± 2.7E5 12.2 1.8	9.6E5 ± 5.1E5 12.7 2.6	7.9E5 ± 6.9E4 10.4 1.9	7.0E5 ± 1.2E5 9.3 1.4	4.1E5 ± 1.0E4 5.4 1.0
Fat	3.6E4 ± 1.1E4 0.5 0.07	5.9E4 ± 2.1E4 0.8 0.2	5.6E4 ± 6.0E3 0.7 0.1	8.9E4 ± 2.7E4 1.2 0.2	6.7E4 ± 1.4E4 0.9 0.2
Blood	5.0E5 ± 1.9E4 6.6 1.0	3.7E5 ± 4.4E4 4.9 1.0	4.2E5 ± 3.8E4 5.6 1.0	5.0E5 ± 5.2E4 6.6 1.0	4.0E5 ± 4.9E4 5.3 1.0
Liver	6.6E5 ± 2.2E5 8.7 1.3	8.8E5 ± 2.2E5 11.6 2.4	1.1E6 ± 9.9E4 14.5 2.6	7.7E5 ± 5.3E3 10.2 1.5	5.7E5 ± 1.8E4 7.5 1.4
Large Intest.	ND[c]	ND	ND	3.1E5 ± 9.1E4 4.1 0.6	2.3E5 ± 1.4E4 3.0 0.6
Small Intest.	ND	ND	ND	3.8E5 ± 3.3E4 5.0 0.8	4.0E5 ± 4.3E4 5.3 1.0
Gall bladder[d]	ND	ND	ND	2.8E5 ± 4.9E4 3.7 0.6	2.5E5 ± 1.2E5 3.3 0.6
Spleen	ND	ND	ND	4.8E5 ± 1.2E4 6.3 1.0	3.6E5 ± 1.1E4 4.8 0.9
Kidney	ND	ND	ND	9.5E5 ± 2.5E5 12.6 1.9	7.0E5 ± 5.7E4 9.3 1.8
Heart	ND	ND	ND	4.4E5 ± 2.5E4 5.8 0.9	3.3E5 ± 1.2E4 4.4 0.8

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg

Table 10.3.3.2. Biodistribution of [4-^{14}C]-VBEEDU at 3, 8, 18, 30 and 60 min post injection of 126 kBq (3.4 μCi) into the jugular vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min
Muscle	2.7E5 ± 5.7E4 3.6[a] 0.5[b]	2.6E5 ± 7.2E4 3.4 0.7	2.5E5 ± 5.6E4 3.3 0.6	3.3E5 ± 1.5E4 4.4 0.9	3.9E5 ± 7.3E4 5.2 1.0
Bone	1.6E5 ± 2.9E4 2.1 0.3	1.7E5 ± 6.3E4 2.2 0.4	1.2E5 ± 3.5E4 1.6 0.3	1.8E5 ± 1.8E4 2.4 0.5	1.9E5 ± 3.5E4 2.5 0.5
Brain	2.7E5 ± 4.2E4 3.6 0.5	1.6E5 ± 4.4E4 2.1 0.4	2.0E5 ± 4.5E4 2.6 0.5	1.5E5 ± 3.1E3 2.0 0.4	1.1E5 ± 5.1E3 1.5 0.3
Lung	7.9E5 ± 4.9E4 10.4 1.5	1.1E6 ± 4.0E5 14.5 2.8	2.1E6 ± 1.1E6 27.8 4.8	5.4E5 ± 1.1E5 7.1 1.5	6.9E5 ± 3.9E5 9.1 1.7
Fat	7.8E4 ± 1.7E4 1.0 0.1	8.0E4 ± 1.8E4 1.1 0.2	1.0E5 ± 1.2E4 1.3 0.2	7.3E4 ± 1.2E4 1.0 0.2	2.5E5 ± 1.6E5 3.3 0.6
Blood	5.4E5 ± 1.6E5 7.1 1.0	3.9E5 ± 1.0E5 5.2 1.0	4.4E5 ± 6.9E4 5.8 1.0	3.6E5 ± 3.1E4 4.8 1.0	4.0E5 ± 6.7E4 5.3 1.0
Liver	4.6E5 ± 1.1E5 6.1 0.9	4.2E5 ± 5.9E4 5.6 1.1	3.1E5 ± 1.1E5 4.1 0.7	5.6E5 ± 7.6E4 7.4 1.6	5.8E5 ± 2.9E4 7.7 1.5

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio

Table 11.3.3.2. Biodistribution of [4-^{14}C]-DVBEEDU at 3, 8, 18, 30 and 60 min post injection of 126 kBq (3.4 μCi) into the jugular vein of Balb-C mice. Data are presented as dpm per gram of wet tissue or mL of blood, as the mean ± SEM (n=3).

Tissue	3 min	8 min	18 min	30 min	60 min
Muscle	4.1E5 ± 8.1E4 5.4[a] 1.0[b]	4.9E5 ± 1.4E5 6.5 1.1	3.0E5 ± 4.4E4 4.0 0.7	4.9E5 ± 4.8E4 6.5 1.3	2.9E5 ± 2.0E4 3.8 1.0
Bone	2.8E5 ± 9.0E4 3.7 0.7	2.5E5 ± 6.6E4 3.3 0.6	1.5E5 ± 5.4E3 2.0 0.4	2.6E5 ± 1.3E4 3.4 0.7	1.8E5 ± 2.7E4 2.4 .06
Brain	3.4E5 ± 3.9E4 4.5 0.8	2.2E5 ± 1.5E4 2.9 0.5	1.1E5 ± 5.0E3 1.5 0.3	2.0E5 ± 1.2E4 2.6 0.5	1.6E5 ± 1.7E4 2.1 0.6
Lung	1.4E6 ± 4.6E5 18.5 3.3	1.4E6 ± 3.6E5 18.5 3.2	1.9E6 ± 4.1E5 25.1 4.5	7.0E5 ± 2.2E4 9.3 1.8	3.3E5 ± 3.2E4 4.4 1.1
Fat	6.3E4 ± 3.4E3 0.8 0.2	1.3E5 ± 4.2E4 1.7 0.3	1.2E5 ± 1.7E4 1.6 0.3	3.2E5 ± 1.9E5 4.2 0.8	1.4E5 ± 1.1E4 1.9 0.5
Blood	4.2E5 ± 4.9E4 5.6 1.0	4.3E5 ± 3.0E4 5.7 1.0	4.2E5 ± 4.1E4 5.6 1.0	3.9E5 ± 4.7E4 5.2 1.0	2.9E5 ± 3.5E4 3.8 1.0
Liver	8.0E5 ± 2.3E5 10.6 1.9	8.6E5 ± 2.5E4 11.4 2.0	6.6E5 ± 1.6E5 8.7 1.6	8.4E5 ± 7.8E4 11.1 2.2	5.0E5 ± 3.3E4 6.6 1.7
Large Intest.	ND[c]	ND	ND	3.9E5 ± 1.5E5 5.2 1.0	2.6E5 ± 4.1E4 3.4 0.9
Small Intest.	ND	ND	ND	6.0E5 ± 1.8E5 7.9 1.5	3.6E5 ± 4.3E4 4.8 1.2
Gall bladder[d]	ND	ND	ND	2.5E5 ± 6.6E4 3.3 0.6	2.6E5 ± 5.0E4 3.4 0.9
Spleen	ND	ND	ND	5.7E5 ± 3.1E4 7.5 1.5	3.1E5 ± 5.1E4 4.1 1.1
Kidney	ND	ND	ND	7.0E5 ± 5.9E4 9.3 1.8	5.5E5 ± 2.5E4 7.3 1.9
Heart	ND	ND	ND	4.9E5 ± 1.6E4 6.5 1.3	2.8E5 ± 3.1E4 3.7 1.0
Urine[e]	ND	ND	ND	5.9E5 ± 1.8E5 7.8 1.5	9.7E5 ± 2.7E5 12.8 3.3

[a]% of injected dose per gram or mL; [b]Tissue/blood ratio; [c]Not determined; [d]Per 100 mg; [e]Activity as dpm/100 μL of urine, based on content of bladder at time of sacrifice

samples taken after jugular injection of [4-^{14}C]-DVBEEDU, compared to those of tail vein injection, radioactivity levels in blood samples taken after either route of injection did not show substantial differences.

3.4. Stability of the 5,6-dihydro prodrugs

Stability of the 5,6-dihydro susbtituents in BEEDU was determined following *in vitro* incubation in several media including normal saline, rat plasma, rat whole blood, gluthatione (GSH) and rat brain homogenate at 37 °C. The results of this study are shown in figure 6.3.4. BEEDU did not undergo conversion to EDU in normal saline. However. this 5,6-dihydro compound was converted to EDU after a 2 hr incubation with rat plasma (8%) and rat brain homogenate (16%). Conversion of BEEDU to EDU following incubation with rat whole blood was more pronounced, with 53% conversion after a 2 hr incubation. In contrast, no regeneration of the 5,6-olefinic bond was observed upon incubation of BEEDU with 2 equivalents of GSH at 37°C for times up to 36 hr.

(5R,6R)-CMEDU and (5S,6R)-CMEDU did not undergo conversion to EDU following a 24 hr *in vitro* incubation with GSH. However, (5R,6R)-BMEDU and (5S,6S)-BMEDU respectively showed 40% and 10% conversion to EDU upon incubation with GSH.

3.5. Biotransformation of BEEDU in rats

The mean concentration-time profiles of BEEDU and EDU in rats which received a 0.55 mmol/kg dose of the test compound are summarized in figure 7.3.5. BEEDU showed a very short blood residence time and its concentration in blood samples taken 18 min post iv injection was below the HPLC detection limit (0.4 μg/mL).

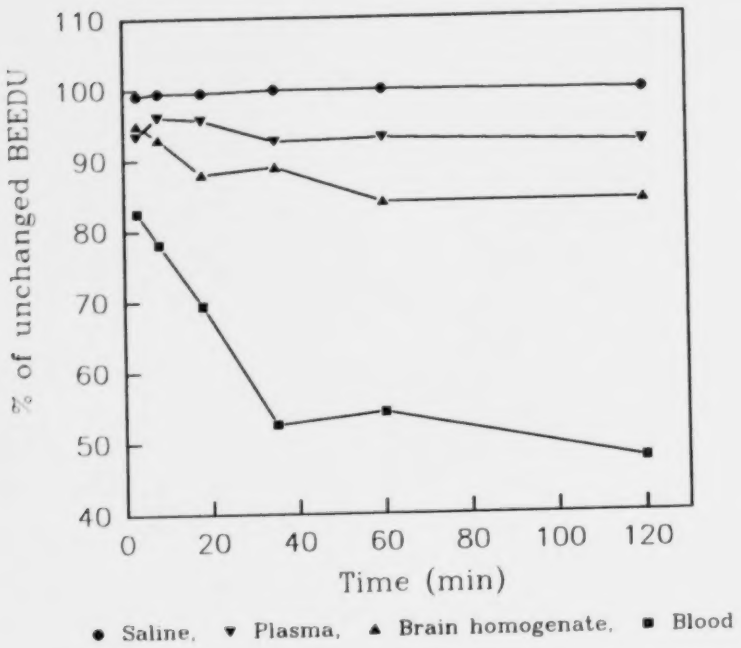


Figure 6.3.4. Stability of BEEDU upon *in vitro* incubation in several media at 37°C.

However, this 5-bromo-6-ethoxy-5,6-dihydro derivative of EDU converted rapidly to EDU after injection and afforded a high concentration of EDU in blood samples. The AUC for EDU, as a metabolite of BEEDU, was much higher (4.25 $\mu mol.hr.mL^{-1}$) than that observed when an equimolar dose of EDU itself was injected (1.83 $\mu mol.hr.ml^{-1}$) (Table 12.3.5). As expected, the metabolism of BEEDU, after conversion to EDU, was similar to that of EDU. EU and HEU were detected as secondary metabolites of BEEDU. The AUC of EU, a metabolite of EDU, was also higher after injection of BEEDU (8.71 $\mu mol.hr.ml^{-1}$) than after injection of EDU (4.35 $\mu mol.hr.ml^{-1}$).

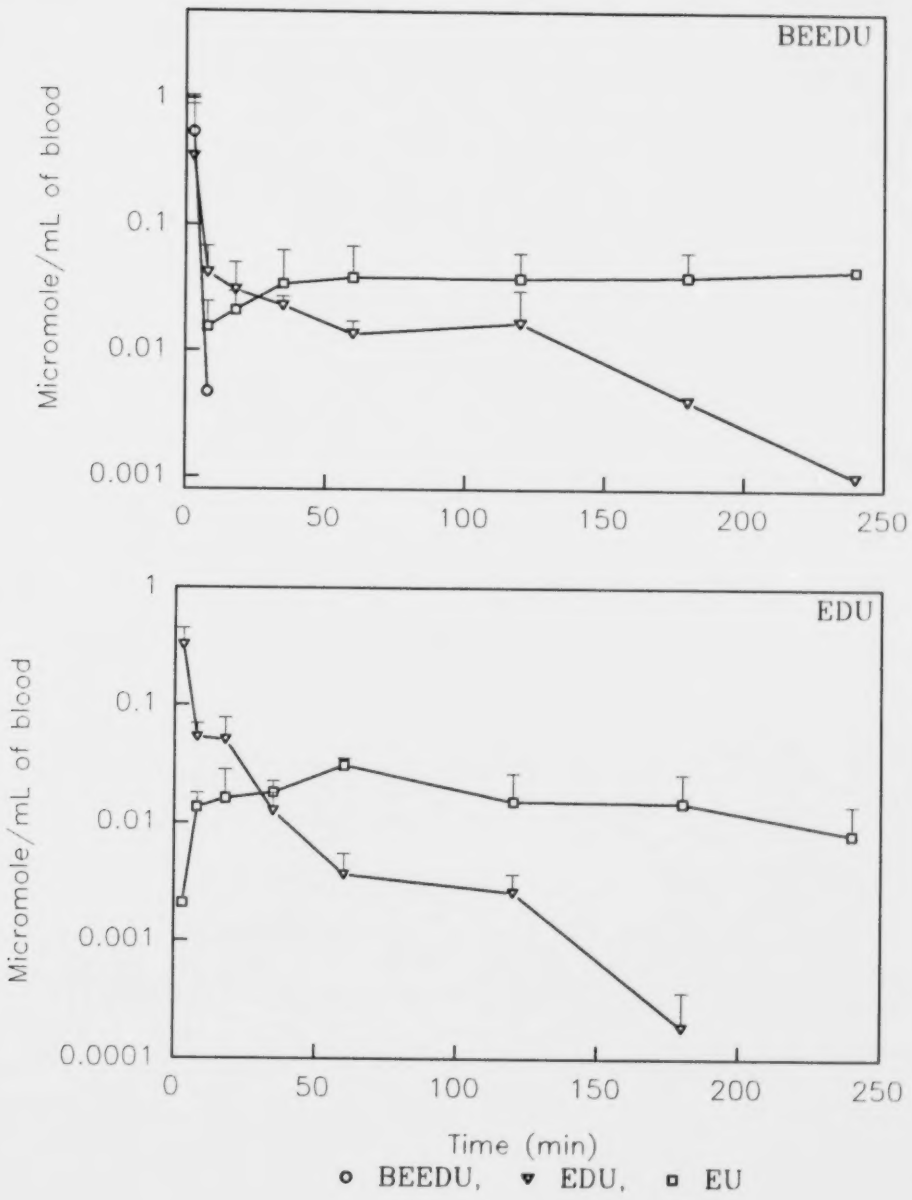


Figure 7.3.5. Blood concentration *vs* time profile of BEEDU and EDU following iv bolus injection of a dose 0.55 mmol/kg into the rats. Data are presented as the mean ± SEM (n=3).

Table 12.3.5. AUC values for EDU and EU after injection of BEEDU or EDU.

Compound injected	AUC[a] (μmol.hr.mL^{-1})	
	EDU	EU
BEEDU	4.25 ± 0.6	8.71 ± 1.3
EDU	1.83 ± 0.4	4.35 ± 1.7

[a]Area under the blood concentration *vs* time curve (0 → last sample). Data are presented as the mean ± SEM (n=3).

3.6. Pharmacokinetics and bioavailability of EDU in mice and rats

The log blood concentration of EDU after an iv dose decreased biexponentially with time (Figure 8.3.6). An excellent fit was obtained using a two compartment model (Figure 9.3.6). Based on this model, the half-lives of the distribution phase, $t_{1/2}\alpha$, in mice and rats were very short (1.4 ± 0.7 min in mice and 1.3 ± 0.1 min in rats). However, the half-lives of the terminal phase, $t_{1/2}\ \beta$, were 24.1 ± 2.9 min in mice and 18.5 ± 1.0 min in rats. Total body clearance (Cl) of EDU was 163.2 ± 10.5 mL.min^{-1}.kg^{-1} in mice and 159.3 ± 30.3 mL.min^{-1}.kg^{-1} in rats. The mean residence time (MRT) for EDU was shorter in rats (11.0 ± 2.9 min) than in mice (25.8 ± 4.9 min) (Table 13.3.6).

Blood concentration data following a po dose were best fit to a one compartment model (Figure 9.3.6). According to this model, the maximum concentration of EDU in blood, C_{max}, was 2.4 ± 0.2 μg/g of blood at a T_{max} of 31.1 ± 1.2 min after dosing. Based on this model, EDU showed a half-life of 50.3 ± 6.2 min in mice. The AUC for EDU after a 100 mg/kg po dose

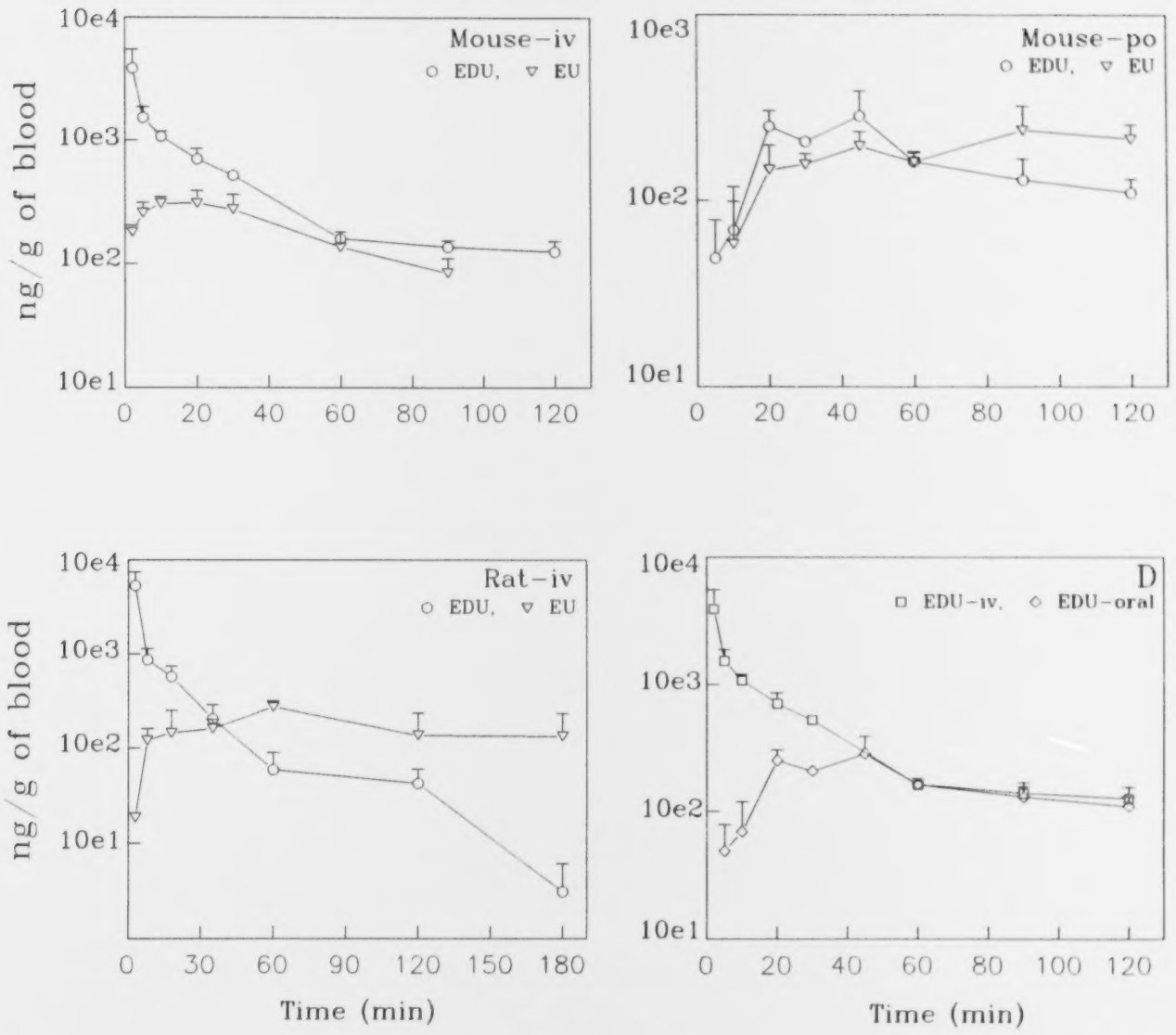


Figure 8.3.6. Blood profile of EDU after a 100 mg/kg iv bolus injection into mice, and rats, and after a 100 mg/kg oral dose in mice. Extent of bioavailability of EDU in mice is shown in plot D. Data are presented as mean ± SD (n=3).

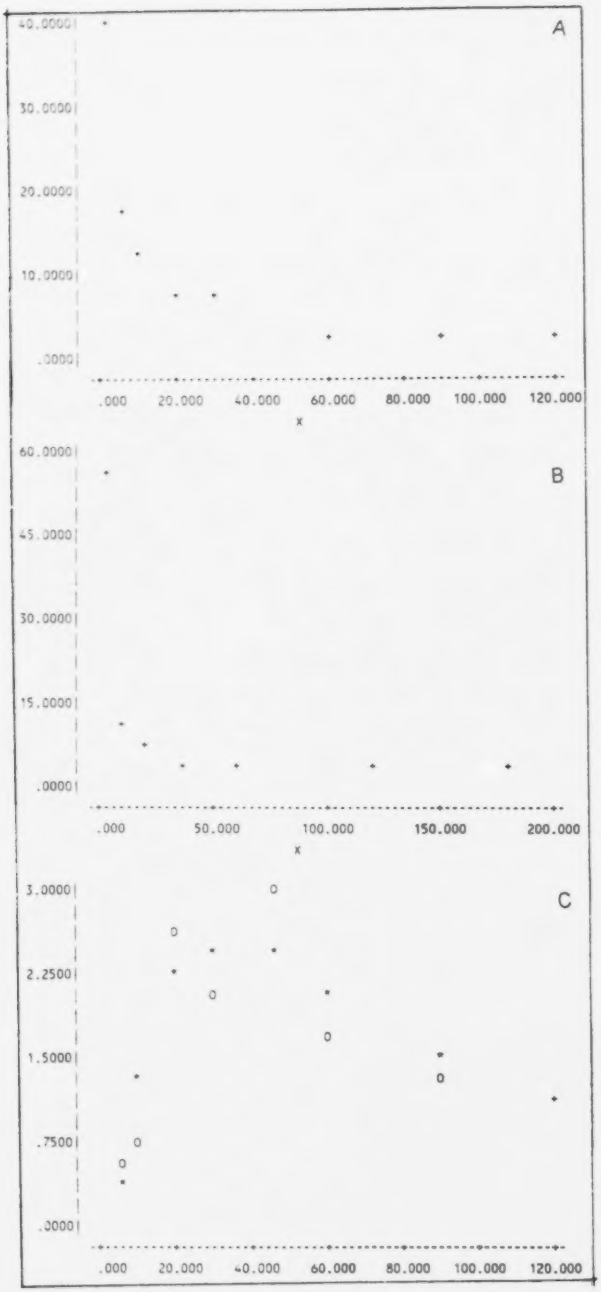


Figure 9.3.6. Plot of observed and calculated values for mean of concentrations of EDU in blood (Y axis) after a 100 mg/kg iv bolus injection into mice (A), rats (B), and after an oral dose into mice (C) *vs* time (X axis).

Table 13.3.6. Pharmacokinetic parameters for EDU after a 100 mg/kg iv bolus injection in mice and rats, based on a two compartment model.

Species	AUC of EDU (μg.g-1.min)	$T_{1/2}\alpha$[a] (min)	$T_{1/2}\beta$[b] (min)	Cl[c] (mL.min-1.kg-1)	MRT[d] (min)	AUC of EU[e] (μg.g-1.min)
Mouse #1	698.71	0.77	18.47	143.12	15.96	224.13
Mouse #2	596.03	0.69	25.67	167.73	29.53	267.01
Mouse #3	559.38	2.86	28.30	178.77	31.96	323.96
mean ± SEM	618.1 ± 41.7	1.4 ± 0.7	24.1 ± 2.9	163.2 ± 10.5	25.8 ± 4.9	249.2 ± 53.8
Rat#1	689.11	1.30	16.53	145.11	5.65	1300.15
Rat#2	459.71	1.37	19.04	217.53	11.54	323.87
Rat#3	866.84	1.10	19.87	115.36	15.86	169.05
mean ± SEM	671.9 ± 117.8	1.3 ± 0.1	18.5 ± 1.0	159.3 ± 30.3	11.0 ± 2.9	597.7 ± 354.1

[a]Half-life of the distribution phase
[b]Half-life of the elimination phase
[c]Blood clearance of EDU
[d]Mean residence time
[e]EU is a metabolite of EDU

Table 14.3.6. Pharmacokinetic parameters for EDU following a 100 mg/kg oral dose in mice based on a one compartment model.

Species	AUC of EDU (μg.g-1.min)	$t_{1/2}$ (min)	T_{max} (min)	C_{max} (μg.g-1)	AUC of EU[a] (μg.g-1.min)
Mouse #1	260.27	38.90	35.59	2.72	1020.13
Mouse #2	267.01	60.30	29.88	2.41	1047.04
Mouse #3	390.31	51.75	29.89	2.06	486.52
mean ± SEM	305.9 ± 42.4	50.3 ± 6.2	31.1 ± 1.2	2.4 ± 0.2	851.2 ± 182.5

[a]EU is a metabolite of EDU

was 305.9 $\mu g.g^{-1}.min$ which indicates a 49% bioavailability for EDU in mice (Table 14.3.6). EU was the major metabolite of EDU after both iv and po dosing, and HEU was detected as a subsequent metabolite of EU. However, the concentration of EU in blood samples taken after a po dose of EDU was significantly higher ($P < 0.05$) than that after an iv dose.

3.7. Enzymatic hydrolysis of VBEEDU

VBEEDU undergoes rapid enzymatic hydrolysis to BEEDU upon incubation with porcine liver esterase at 37°C (Table 15.3.7), since more than 95% of VBEEDU was hydrolyzed after an 8 min incubation. No chemical hydrolysis of VBEEDU was observed using a control experiment not containing the enzyme. This observation indicates that VBEEDU is chemically stable in aqueous solution.

Table 15.3.7. Enzymatic hydrolysis of VBEEDU upon incubation with porcine liver esterase. Data are the means of two separate experiments.

Incubation time (min)	% unhydrolyzed VBEEDU[a]
3	26
8	4.3
18	0.8

[a]The only hydrolysis product detected by HPLC was BEEDU.

3.8. *In vitro* and *in vivo* anti-HSV activity of EDU, BEEDU and VBEEDU

In vivo ability of these compounds to protect NMRI mice inoculated intracerebrally with HSV-1 and HSV-2 and *in vitro* antiviral activities of EDU, BEEDU and VBEEDU against different strains of HSV in Hela cells are summarized in Tables 16.3.8 and 17.3.8. The overall anti-HSV potency and activity spectra for BEEDU and VBEEDU were similar to that of EDU (Table 17.3.7). Neither EDU nor the prodrugs BEEDU and VBEEDU protected NMRI mice inoculated intracerebrally with HSV-1 or HSV-2 (Table 16.3.8).

3.9. Bioavailability and pharmacokinetics parameters for BEEDU and VBEEDU in mice

The mean concentration-time profiles of VBEEDU and BEEDU in mice which received a 0.4 mmol/kg iv dose of the test compound are summarized in figure 10.3.8. BEEDU showed a very short blood residence time since virtually no BEEDU was detected in blood samples taken 10 min post iv injection. This result is due to the fact that BEEDU is rapidly biotransformed to EDU in mice to afford a high concentration of EDU in blood samples (Figure 10.3.8). The AUC for EDU, a metabolite of BEEDU, was higher (2.1 ± 0.3 $\mu mol.hr.g^{-1}$) than that observed when EDU was injected (1.7 ± 0.2 $\mu mol.hr.g^{-1}$) (Table 18.3.8). VBEEDU provided a consistently high concentration of EDU in blood following iv injection into mice. The AUC for EDU, a metabolite of VBEEDU, was 1.8 ± 0.2 $\mu mol.hr.g^{-1}$. In contrast to BEEDU which could not be detected at 10 min post injection, VBEEDU and 5'-O-valeryl-5-ethyl-2'-deoxyuridine (VEDU) were detected in blood samples collected up to 35 min post injection. The metabolism of VBEEDU and BEEDU, after conversion to EDU, was similar

Table 16.3.8. Intraperitoneal treatment of intracerebral HSV-1 or HSV-2 infected NMRI mice using EDU, BEEDU, VBEEDU and acyclovir.

	Survival rate at the 20th day	
Compound	HSV-1[a]	HSV-2[b]
EDU (0.78 mmol/kg/day)	0/5	0/5
EDU (0.20 mmol/kg/day)	0/5	0/5
BEEDU (0.78 mmol/kg/day)	0/5	0/5
BEEDU (0.20mmol/kg/day)	0/5	0/5
VBEEDU (0.78 mmol/kg/day)	0/5	0/5
VBEEDU (0.20 mmol/kg/day)	0/5	0/5
Acyclovir (0.89 mmol/kg/day)	3/5	2/5
Acyclovir (0.22 mmol/kg/day)	2/5	0/5
Placebo (PBS) (30% DMSO)	0/5	0/5

[a]Five twenty-five-day old NMRI mice (weighing 11-13 g) were inoculated intracerebrally with HSV-1(KOS) at 3 $CCID_{50}$/0.02 mL/mouse and treated intraperitoneally with the test compound twice a day for 5 days, starting on the day of virus infection. Compounds were dissolved in phosphate-buffered saline (PBS) containing 30% DMSO.

[b]Five twenty-five-day old NMRI mice (weighing 11-13 g) were inoculated intracerebrally with HSV-2(196) at 0.3 $CCID_{50}$/0.02 mL/mouse and treated intraperitoneally with the test compound twice a day for 5 days, starting on the day of virus infection. Compounds were dissolved in phosphate-buffered saline (PBS) containing 30% DMSO.

Table 17.3.8. *In vitro* activity of EDU, BEEDU and VBEEDU against different HSV strains in Hela cells.

Compound	Cytotoxicity (μM)		Minimum inhibitory concentration[a] (μM)									
	cell growth (CC_{50})[b]	cell morphology (MIC)[c]	HSV-1 (KOS)	HSV-1 (F)	HSV-1 (McIntyre)	HSV-2 (G)	HSV-2 (196)	HSV-2 (Lyons)	HSV-1 TK^- (B2006)	HSV-1 TK^-/TK^+ (VMW1837)	Vaccinia virus	Vesicular stomatitis virus
EDU	150	>200	10	20	5	10	5	5	>200	>200	2	>200
BEEDU	85	>200	5	2	5	5	5	5	>200	100	2.7	>200
VBEEDU	75	>100	6.5	19	3	7.5	21	7.5	>100	86	5.3	>100
Acyclovir	840	>150	0.01	0.0084	0.0054	0.0126	0.017	0.0084	38	8.4	>200	>200
BVDU	300	>150	0.01	0.0093	0.0039	>150	>150	>150	150	>150	6	>150

[a]Concentration required to reduce virus-induced cytopathogenicity by 50%.
[b]Concentration required to reduce cell growth by 50% after 3 days of incubation.
[c]Minimum cytotoxic concentration that causes a microscopically detectable alteration of normal cell morphology after 2 days of incubation.

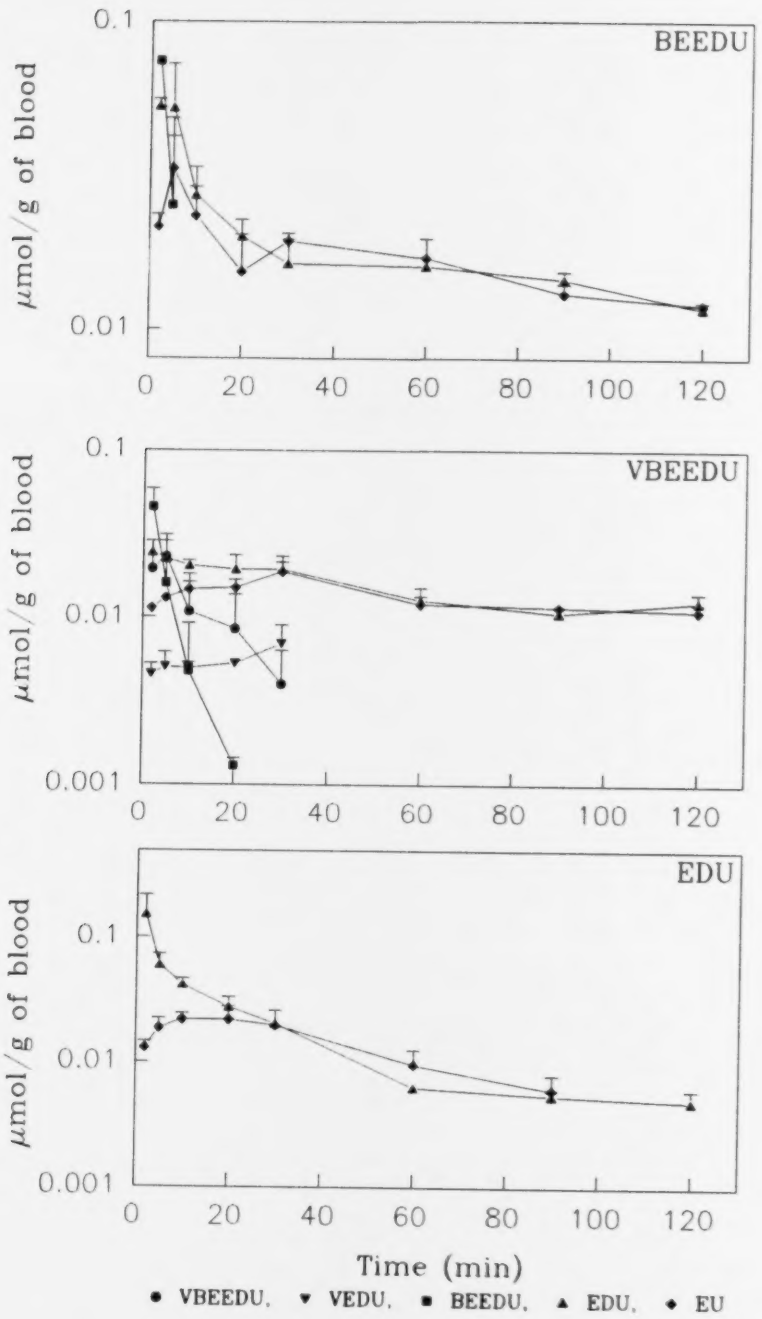


Figure 10.3.8. The mean blood concentration-time profiles after injection of BEEDU, VBEEDU and EDU in mice. Data are presented as the mean ± SD (n=3).

to that of EDU. EU and HEU were detected as secondary metabolites of EDU, BEEDU and VBEEDU.

The blood profiles for EDU and EU, after iv and po administration and extent of bioavailability of EDU following po administration EDU, BEEDU and VBEEDU are shown in figures 11.3.8 and 12.3.8. The pharmacokinetic parameters for EDU after iv and po administration of EDU, BEEDU and VBEEDU are summarized in Table 16.3.8. EDU was eliminated from blood with a half-life of 35.2 ± 4.2 min. The MRT and AUC for EDU after iv injection were 45.1 ± 11.7 min and 1.7 ± 0.2 $\mu mol.g^{-1}.min$, respectively. The AUC for EDU, a metabolite of BEEDU, after a 0.4 mmol/kg iv dose of BEEDU was 2.1 ± 0.3 $\mu mol.g^{-1}.min$ which is substantially higher than that observed after iv injection of EDU (Table 16.3.8). The half-life and MRT of EDU were increased to 251.9 ± 30.2 min and 352.0 ± 91.5 min, respectively after injection of BEEDU. The oral bioavailability of EDU, after administration of BEEDU, was increased almost 2 fold (81%) compared to that of after administration of EDU (49%). However, the AUC_{po} for EDU after oral administration of the 5,6-dihydro prodrug BEEDU (1.7 ± 0.2 $\mu mol.g^{-1}.min$) was equal to that of EDU after iv injection of EDU. This represents a 100% (h, Table 18.3.8) oral availability for EDU after administration of BEEDU. The half-life and MRT of EDU after po administration of BEEDU were 165.9 ± 23.2 min and 234.5 ± 60.9 min respectively. These values were lower than the corresponding values after iv injection of BEEDU. The AUC of EDU after iv injection of VBEEDU was 1.8 ± 0.2 $\mu mol.g^{-1}.min$. The half-life and MRT of EDU, a metabolite of VBEEDU, were 106.9 ± 23.2 min and 157.0 ± 40.8 min which are substantially higher than those for EDU after administration of EDU. Oral administration of VBEEDU provided an 89% bioavailability for EDU.

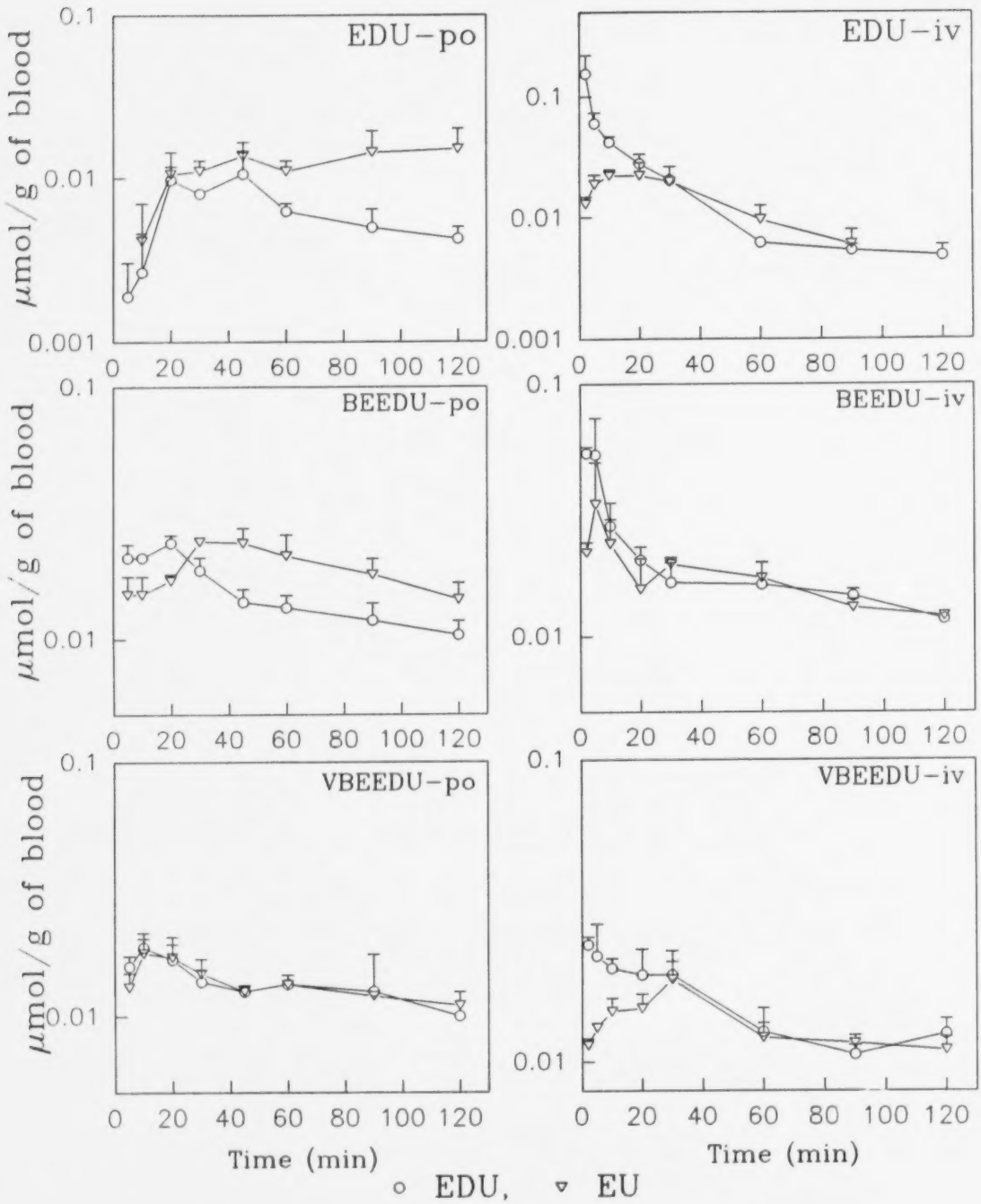


Figure 11.3.8. Blood profile of EDU and EU after po and iv administration of EDU, BEEDU and VBEEDU into mice. Data are presented as the mean ± SD (n=3).

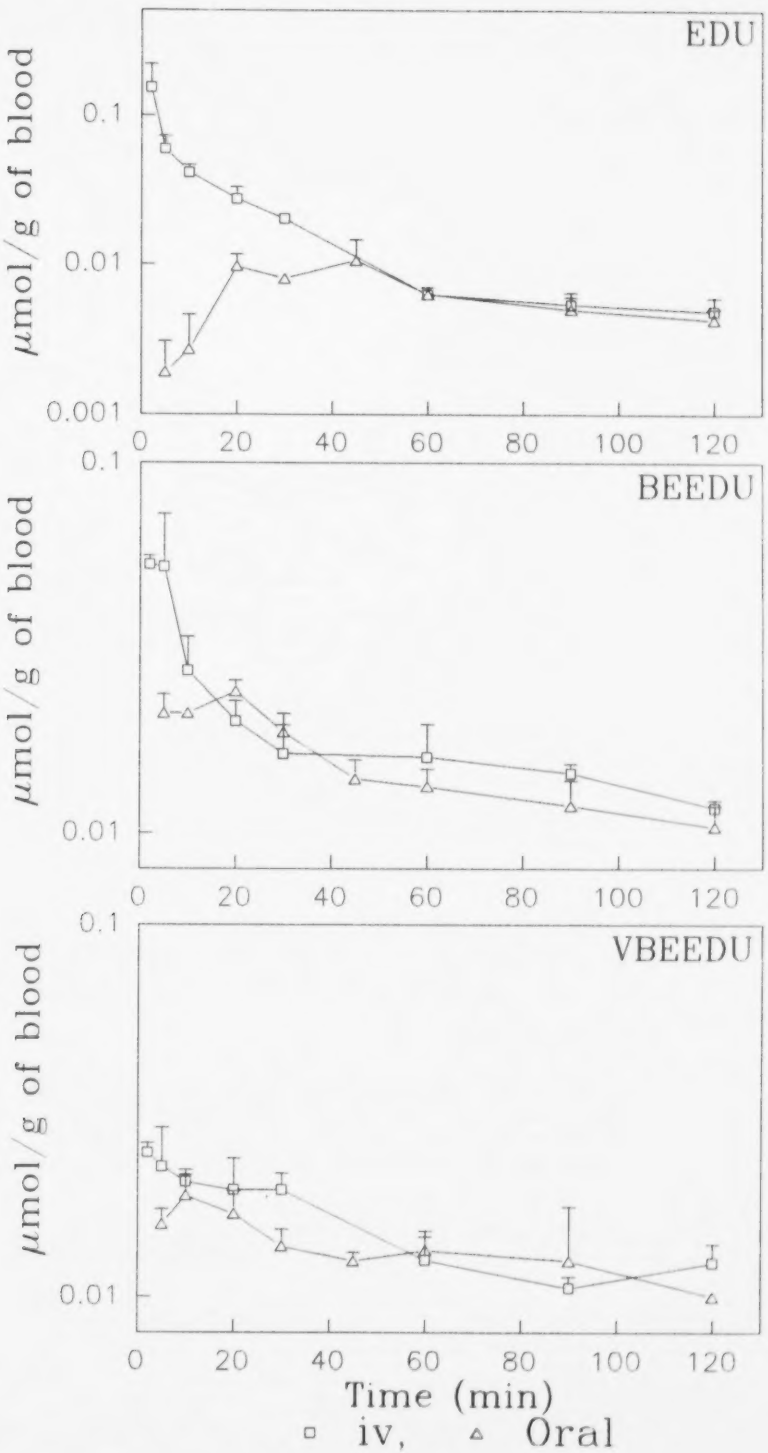


Figure 12.3.8. Blood concentration of EDU following iv or po administration of EDU, BEEDU and VBEEDU in mice. Values are the means ± SD (n=3).

Table 18.3.8. Pharmacokinetic parameters and oral bioavailability of EDU after iv and po administration of 0.4 mmol/kg of EDU, BEEDU and VBEEDU in male Balb-C mice. Values are the means ± SD (n=3).

Parameter	EDU_{iv}	EDU_{po}	$BEEDU_{iv}$	$BEEDU_{po}$	$VBEEDU_{iv}$	$VBEEDU_{po}$
AUC[a] (μmol.g^{-1}.min)	1.7 ± 0.2	0.7 ± 0.1	2.1 ± 0.3[b]	1.7 ± 0.2[b]	1.8 ± 0.2[c]	1.6 ± 0.2[c]
	1.2 ± 0.2[d]	1.3 ± 0.1[d]	1.9 ± 0.2[d]	2.0 ± 0.3[d]	1.6 ± 0.3[d]	1.6 ± 0.3[d]
$t_{1/2}$ (min)	35.2 ± 4.2	49.8 ± 6.0	251.9 ± 30.2	165.9 ± 23.2	106.9 ± 23.2	247.4 ± 29.6
MRT[e] (min)	45.1 ± 11.7	92.4 ± 24.0	352.0 ± 91.5	234.5 ± 60.9	157.0 ± 40.8	356.9 ± 92.6
f[f]	49%	-	81%	-	89%	-
h[g]	-	-	-	100%		94%
Ratio of AUCs[h]	0.7	1.8	0.9	1.2	0.9	1.0

[a]Area under the blood concentration curve *vs* time (0 → 120 min).
[b]AUC of EDU after iv or po administration of BEEDU.
[c]AUC of EDU after iv or po administration of VBEEDU.
[d]AUC of EU a metabolite of EDU.
[e]Mean residence time.
[f]$f=(AUC_{po}/AUC_{iv}) \times 100$.
[g]Systemic availability of EDU based on the AUC for EDU after po administration of the prodrug/AUC of EDU after iv injection of EDU.
[h]AUC(EU)/AUC(EDU).

However, the availability of EDU, using the AUC_{po} for EDU after po administration of VBEEDU and the AUC_{iv} for EDU after iv injection of EDU, was 94% (h, Table 18.3.8). EU was a major metabolite of EDU after both iv and po administration of EDU or the 5-bromo-6-ethoxy-5,6-dihydro prodrugs BEEDU and VBEEDU. The ratio of the AUC(EU)/AUC(EDU) after po administration of EDU (1.8) was significantly higher than after iv injection (0.7). This ratio was also higher after administration of either BEEDU or VBEEDU.

However, differences between ratios of AUC(EU)/AUC(EDU) after both iv and po administration of BEEDU and VBEDU were smaller than the differences between AUC(EU)/AUC(EDU) after iv and po dose of EDU.

3.10. Analysis of lung samples after injection of EDU, BEEDU and VBEEDU

The distribution of radioactivity after injection of 126 kBq (3.4 μCi) mixed with 0.2 mmol/kg of non radioactive test compound of either EDU or its 5-bromo-5-ethyl-6-alkoxy-5,6-dihydro prodrug (BMEDU diastereomers, BEEDU, VBEEDU and DVBEEDU) is summarized in figure 13.3.9. EDU showed a rapid distribution into liver and lung shortly after injection, and the level of radioactivity was slightly higher than that in blood. However, the overall distribution of radioactivity in blood, liver and lung was similar. Samples taken 3 min after injection of [4-^{14}C]-EDU showed 9.6, 9.7 and 8.1% of the injected radioactivity per gram of lung, liver and mL of blood, respectively. The distribution of radioactivity in lung after injection of [4-^{14}C]-(5S,6S)-BMEDU and [4-^{14}C]-(5R,6R)-BMEDU was substantially different from each other, and also from that of [4-^{14}C]-EDU. Lung samples taken 8 min post injection of [4-^{14}C]-(5S,6S)-BMEDU showed the highest level of radioactivity. However, injection of [4-^{14}C]-(5R,6R)-BMEDU provided the highest radioactivity in lung at 3 min post injection. The radioactivity level present in lung samples after injection of both [4-^{14}C]-(5S,6S)-BMEDU and [4-^{14}C]-(5R,6R)-BMEDU was substantially higher than that of blood samples. Injection of [4-^{14}C]-BEEDU into mice provided a higher radioactivity in lung samples compared to those of EDU and BMEDU diastereomers. Radioactivity present in lung samples taken 18 min after injection of [4-^{14}C]-BEEDU was significantly higher ($P < 0.05$) than in liver

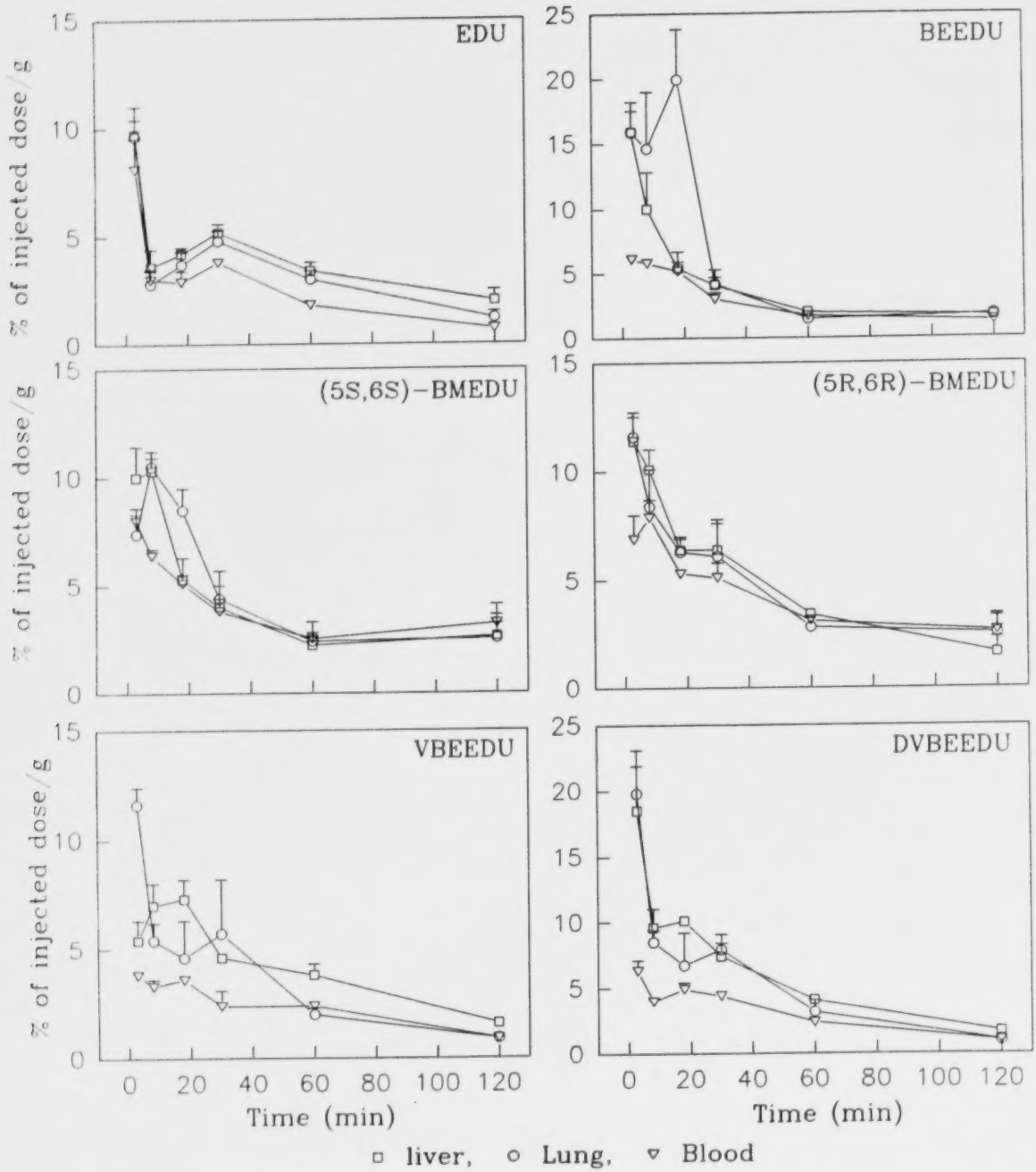


Figure 13.3.9. Percent of injected dose present per gram of lung, liver and blood samples after iv injection of 126 kBq (3.4 μCi) of [4-^{14}C]-EDU, [4-^{14}C]-BEEDU, [4-^{14}C]-(5S,6S)-BMEDU, [4-^{14}C]-(5R,6R)-BMEDU, [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU into mice. Values are the mean ± SEM (n=3).

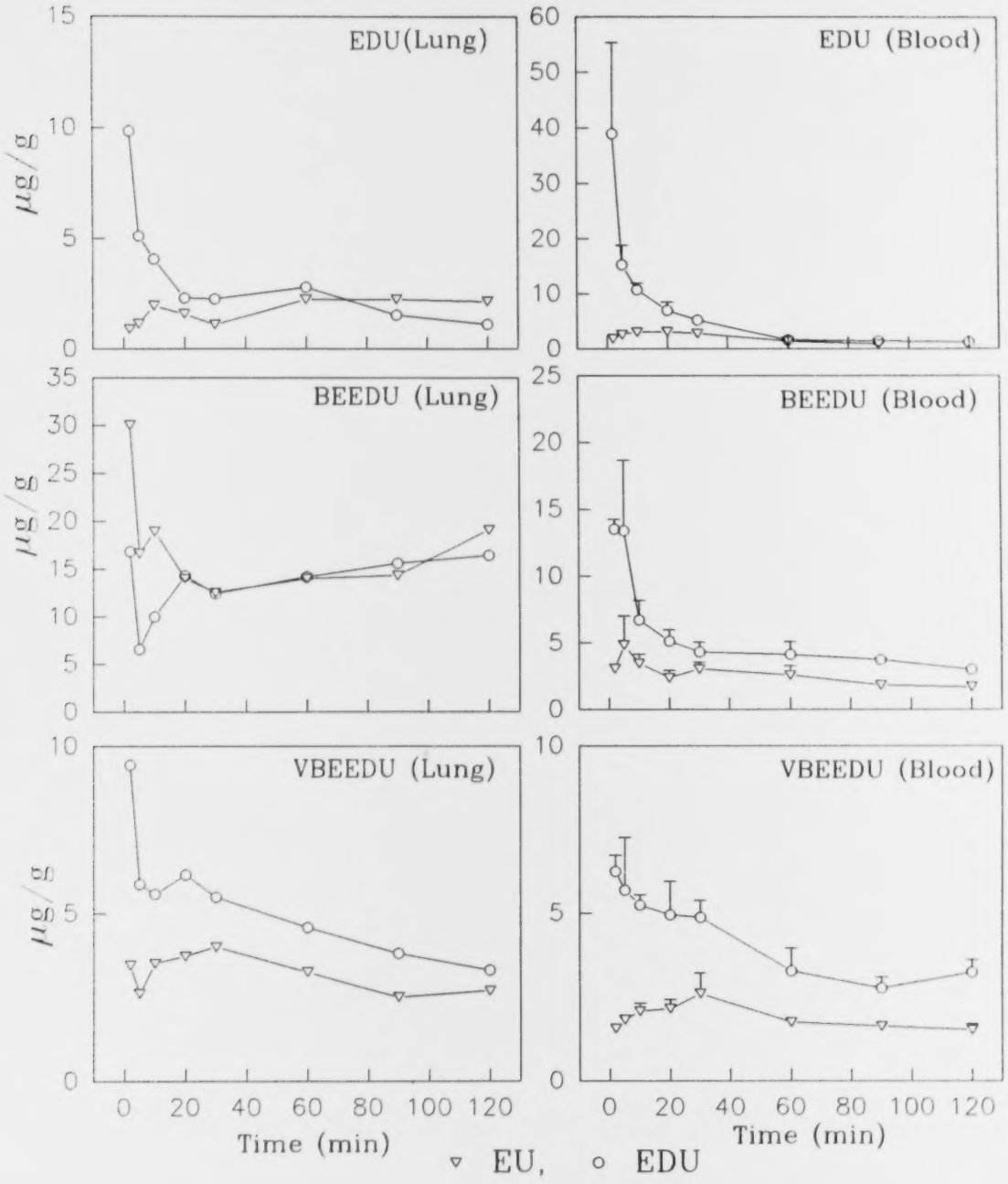


Figure 14.3.9. Concentration of EDU and EU (μg/g) in blood and lung samples after iv injection of a 0.4 mmol/kg dose of EDU, BEEDU and VBEEDU into mice. Values are the mean ± SEM (n=3) where applicable.

and blood samples at this time interval. Although the radioactivity present in lung samples after injection of [4-^{14}C]-VBEEDU was significantly higher ($P < 0.05$) than those in liver and blood samples, [4-^{14}C]-VBEEDU did not provide higher radioactivity in lung samples than [4-^{14}C]-BEEDU. The radioactivity level present in lung samples after injection of DVBEEDU was similar to that of liver samples. However, lung samples showed a considerably higher percent of the injected dose than did blood samples.

The HPLC analysis results for lung and blood samples after injection of a 0.4 mmol/kg iv dose of either EDU, BEEDU or VBEEDU into mice are shown in figure 14.3.9. It appears that EDU is the major component present in lung samples. Although the concentration of EDU in lung samples taken 2 min after injection of EDU was substantially lower than that of blood, the concentration of EDU in lung samples obtained at longer times was very similar to that of blood samples. In contrast to lung samples, EU was not detected in blood samples taken at 120 min post injection of EDU. The concentrations of both EDU and EU in lung samples after injection of BEEDU was substantially higher than that of blood samples. In contrast to blood samples, both EDU and EU showed accumulation in lung samples following injection of BEEDU. Lung samples taken after injection of VBEEDU also showed a considerably higher concentration of EDU and EU compared to that of EDU.

3.11. Biliary excretion of [4-^{14}C]-labelled (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU

Distribution of radioactivity after injection of 126 kBq (3.4 μCi) of [4-^{14}C]-BEEDU, [4-^{14}C]-(5S,6S)-BMEDU, [4-^{14}C]-VBEEDU or [4-^{14}C]-DVBEEDU, mixed with 0.2 mmol/kg of non radioactive test compound, in

blood, liver, large intestine, small intestine and gall bladder of male Balb-C mice was discussed in section 3.3.1. All of these 5-bromo-6-alkoxy-5,6-dihydro derivatives of EDU , in most samples, provided a higher percentage of the injected dose in liver than in blood. A substantial amount of radioactivity was also present in large intestine, small intestine and gall bladder. Intestine samples taken at longer times post injection of the test compound showed a higher percentage of the injected dose than those taken at shorter time intervals.

Biliary excretion of the [4-^{14}C]-labelled (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU after a bolus injection of 112 kBq (3.0 μCi) mixed with 0.2 mmol/kg of non radioactive test compound into jugular vein of rats having a catheter in bile duct, are shown in Figure 15.3.10 and 16.3.10. Bile samples that were collected 8 min post injection of these 5,6-dihydro prodrugs showed the highest radioactivity levels. Excretion of radioactivity in bile showed a biexponential decline. However, the excretion rates of radioactivity in bile after injection of all four 5-bromo-6-alkoxy-5,6-dihydro prodrugs to EDU were quite similar (Figure 3A). Accumulation of radioactivity in bile samples collected after injection of [4-^{14}C]-BEEDU was substantially higher than after injection of [4-^{14}C]-(5S,6S)-BMEDU, [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU (Figure 16.3.10).

3.12. Blood/plasma ratios of [4-^{14}C]-labelled (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU

Blood and plasma samples collected following injection of [4-^{14}C]-labelled (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU at the same post injection times showed substantially different levels of radioactivity (Figure 15.3.10). The ratios of whole blood / plasma radioactivity levels for

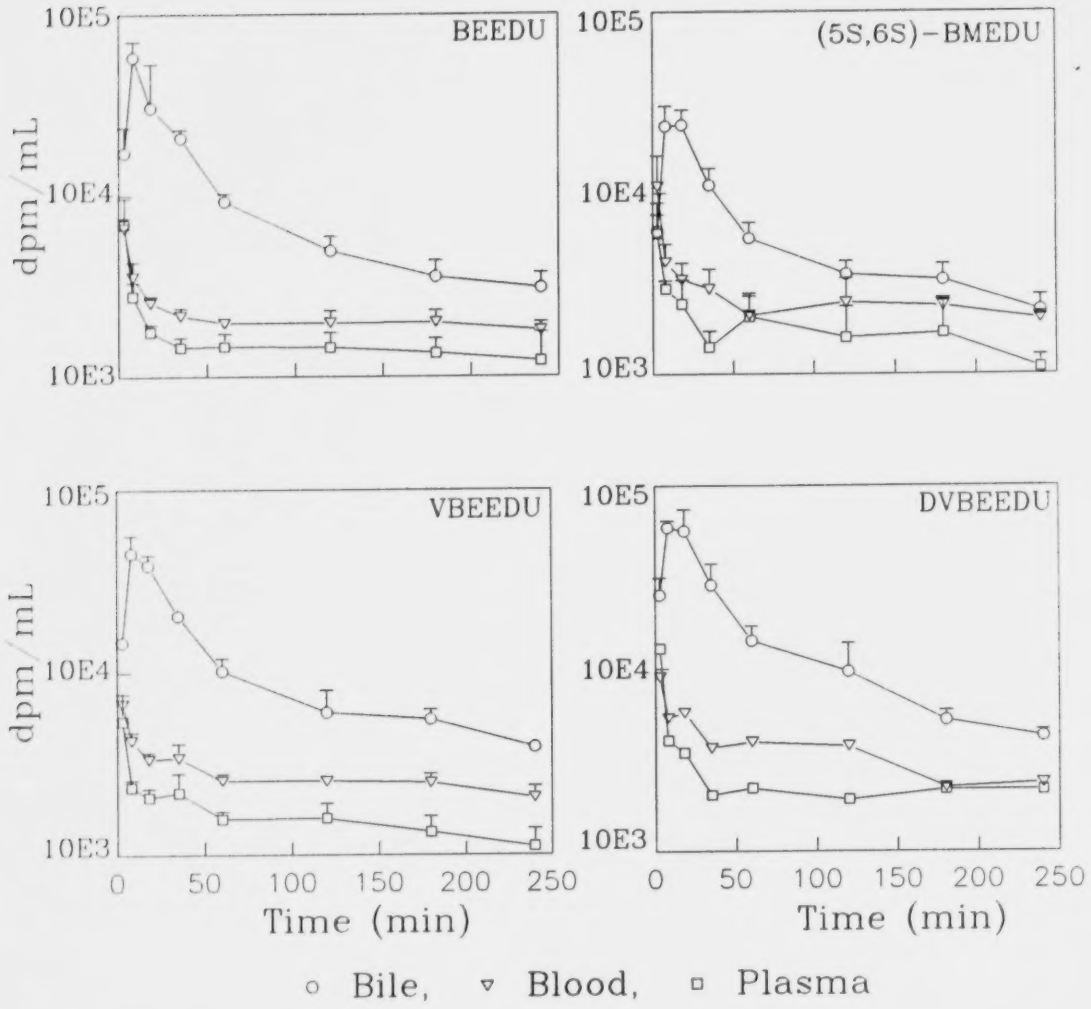


Figure 15.3.10. Biliary excretion of radioactivity and differences between the distribution of radioactivity in whole blood and plasma after injection of [4-^{14}C]-labelled BEEDU, (5S,6S)-BMEDU, VBEEDU, and DVBEEDU into rats. Values are the mean ± SD (n=3, except where only one rat was used for measurement of radioactivity levels in whole blood and plasma samples of DVBEEDU).

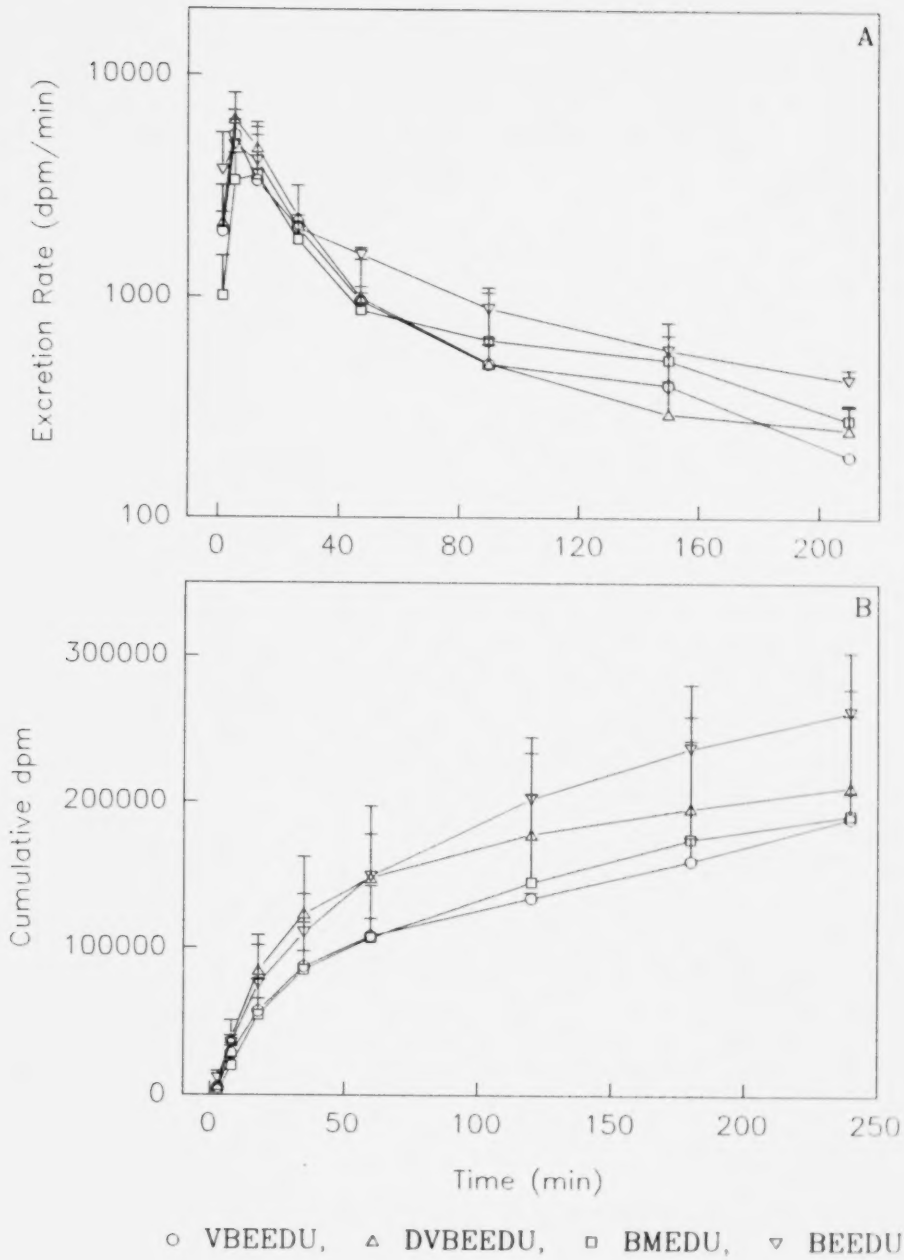


Figure 16.3.10. Excretion rates (A) and cumulative excretion of radioactivity in bile samples (B) after injection of [4-^{14}C]-(5S,6S)-BMEDU, [4-^{14}C]-BEEDU, [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU into rats. Values are the means ± SD (n=3).

the mean radioactivity levels in blood or plasma samples, for these prodrugs, are also shown in Table 19.3.11. Radioactivity levels in blood samples taken at 8 min or longer post injection of [4-^{14}C]-VBEEDU showed significantly ($P < 0.05$) higher radioactivity levels than in the corresponding plasma samples. Although blood samples collected 18 min post injection of [4-^{14}C]-BEEDU showed significantly ($P < 0.05$) higher radioactivity levels than those for the corresponding plasma samples, radioactivity levels in blood samples collected at time intervals shorter than 35 min post injection of [4-^{14}C]-BMEDU did not show significant differences compared to plasma samples.

All the blood samples taken at > 3 min post injection for all of these 5,6-dihydro prodrugs showed higher radioactivity levels than those of corresponding plasma samples. However, blood samples taken at 3 min post injection of [4-^{14}C]-BEEDU and [4-^{14}C]-DVBEEDU showed lower radioactivity levels than those of corresponding plasma samples. The lowest whole blood/plasma ratio of radioactivity level was observed after injection of [4-^{14}C]-BEEDU compared to those after injection of [4-^{14}C]-(5S,6S)-BMEDU, [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU.

Table 19.3.11. Whole blood / plasma ratios of radioactivity levels for the means of radioactivity levels in blood and plasma samples, after iv injection of [4-^{14}C]-labelled (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU into rats.

	Whole blood / plasma ratios			
	Compound injected			
Time (min)	(5S 6S)-BMEDU	BEEDU	VBEEDU	DVBEEDU
3	1.82	0.95	1.23	0.70
8	1.4	1.27	1.81	1.33
18	1.37	1.45	1.62	1.65
35	2.10	1.49	1.56	1.82
60	1.02	1.34	1.74	1.80
120	1.56	1.37	1.61	1.98
180	1.41	1.48	1.88	1.02
240	1.85	1.47	1.86	1.09

4. Discussion

4.1. Catabolism and anabolism of antiviral pyrimidine nucleosides

Antiviral nucleosides interact with several enzymes during their metabolic transformation. First, they have to be converted to an active nucleotide form to exert their antiviral effect(s). However, nucleosides are also substrates for several enzymes which are responsible for their degradation. There are at least eight enzymes which have important interactions with antiviral nucleosides. Interaction of antiviral nucleosides with some of these enzymes results in toxicity to the host cell. Enzymes which their interactions with antiviral nucleosides are undesirable include: nucleoside phosphorylase, cellular TK, thymidylate synthase (for 5'-monophosphate analogs) and cellular DNA polymerase. Nucleoside phosphorylase inactivates the nucleoside by glycosidic bond cleavage and reaction with latter enzymes listed above would cause cell toxicity. However, phosphorylation of antiviral nucleosides by viral TK, viral thymidylate kinase, nucleoside diphosphate kinase and viral DNA polymerase activate the nucleoside. For example, viral TK, which catalyzes the phosphorylation of antiviral nucleosides, plays a crucial role in activation of the nucleoside.

HSV-TK is capable of phosphorylating deoxythymidine, deoxycytidine and related nucleoside analogs to their respective monphosphates. HSV-TK also has thymidylate kinase activity and it can convert TMP to TDP (De Clercq et al 1988). It is well-known that HSV-TK is the reason for the selectivity of acyclovir, and many other nucleoside analogs, since they are phosphorylated efficiently by the virus TK, but not by mammalian kinase. Once the nucleoside monphosphates are formed, they are converted to their triphosphates by cellular and/or virus enzymes

prior to their interaction with virus DNA-polymerase (De Clercq et al 1988). It was reported that both human TK and HSV-TK consist of a central core β-sheet structure, connected by loops and α-helices very similar to the overall structure of other nucleotide binding enzymes. The phosphate binding site is comprised of a highly conserved glycine-rich loop at the N-terminus of the protein and a conserved region at the C-terminus. The thymidine recognition site is located about 100 amino acids downstream from the phosphate binding loop. The differing substrate specificity of human and HSV-1 TK may be explained by amino-acid substitutions in homologous regions (Folkers et al 1991).

EDU, a pyrimidine nucleoside, is metabolized by both anabolic and catabolic pathways which lead to the formation of an active nucleotide and an inactive 5-ethyluracil base, respectively. Anabolic metabolism is involved in the conversion of EDU to EDUTP, which is responsible for the antiviral effects of the drug. The initial phosphorylation to EDUMP is catalyzed by a HSV-TK. Since EDU is a much poorer substrate for the host cell TK than the HSV-TK, it is selectively activated in virus-infected cells (De Clercq et al 1987). Host cell nucleoside monophosphate kinases and diphosphate kinases catalyze the further phosphorylation of EDUMP to EDUTP. The antiviral activity exhibited by EDUTP is due to its ability to inhibit HSV DNA polymerase. The subsequent metabolic reactions involved in the antiviral action of EDU are summarized in figure 1.4.1. The selectivity of EDU as an anti-herpes agent depends on its preferential phosphorylation by the virus-TK and the preferential incorporation of EDUTP into viral DNA. The latter event is then followed by suppression of viral DNA synthesis and the formation of viral progeny. A number of enzymatic reactions reduce the ability of EDU to achieve its selective inhibitory effect on virus replication

such as its premature degradation by pyrimidine nucleoside phosphorylases.

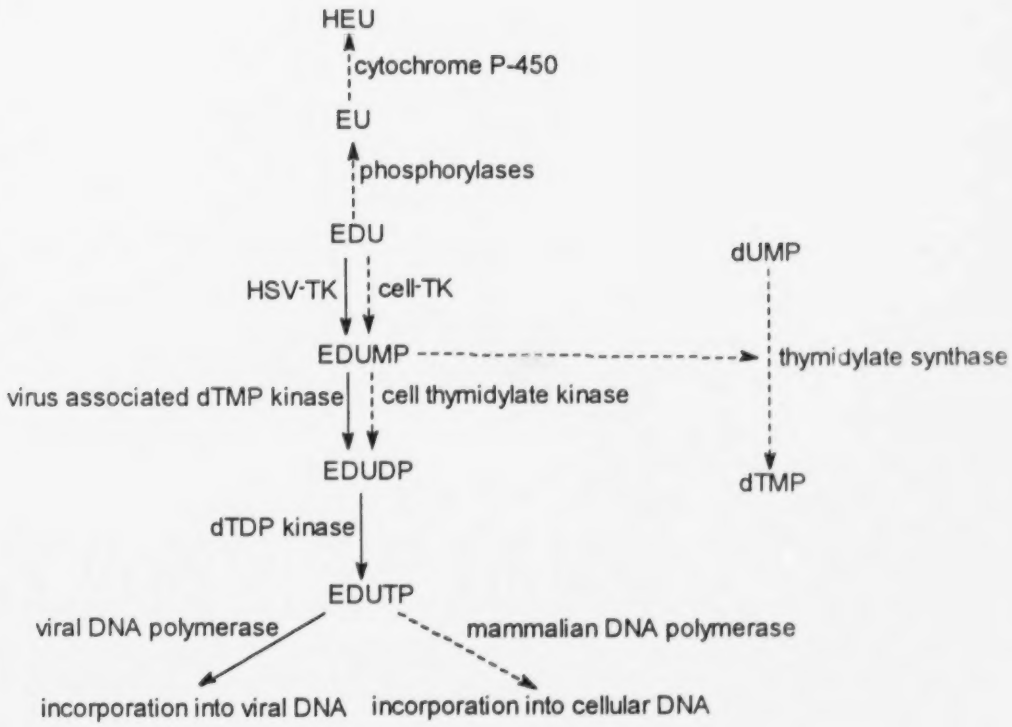


Figure 1.4.1. Mechanism of action and possible enzymatic reactions of EDU. Solid lines indicate desired reactions and dotted lined show undesirable interactions. Inhibition of cellular DNA synthesis by EDU, or inhibition of dTMP synthase by EDUMP, can only occur at 50 μM EDU concentration which is far in excess of the minimum effective dose required to inhibit viral progeny formation (De Clercq et al 1987).

Pyrimidine nucleosides first undego catabolism by phosphorylases which cleave the glycosidic bond. There are two different pyrimidine phosphorylases in mammalian cells: uridine phosphorylase and thymidine phosphorylase (Veres et al 1988). These enzymes catalyze the following reversible reaction:

pyrimidine nucleoside + inorganic phosphate ↔ uracil base + deoxyribose-1-phosphate

In addition to their role as salvage enzymes in pyrimidine metabolism, pyrimidine nucleoside phosphorylases are responsible for the catabolism of a number of 5-substituted pyrimidine nucleoside analogs that exhibit antiviral or chemotherapeutic activity. The uracil bases are then degraded in the liver primarily to carbon dioxide, ammonia and β-alanine. The degradative route for pyrimidine nucleosides proceeds via uracil or thymine. These two pyrimidine bases share a common degradative pathway which utilize the same enzymes (Levine et al 1974).

The initial step in the catabolism of EDU involved a reaction with inorganic phosphate to form EU and 2-deoxyribose-1-phosphate. Although both uridine and thymidine phosphorylases could catabolize EDU (Joly et al 1991b), it was reported that EDU is a better substrate for uridine phosphorylase (Veres et al 1988). It was reported that uridine phosphorylase of rat intestinal mucosa catabolyses EDU better than thymidine phosphorylase from mouse liver (Veres et al 1986). Cleavage of the glycosidic bond of EDU is followed by hydroxylation of the 5-ethyl group, most likely by cytochrome P-450 enzymes (Joly et al 1991b).

Although there is a similarity in the chemical structures of EDU, FUDR and deoxythymidine, there are differences in the metabolic fates of

the bases derived from these nucleosides. The endogenous uracil bases, uracil and thymine, and the structurally related FU, undergo rapid and extensive catabolism by a common metabolic pathway (Heidelberger 1975, Wasternack 1980, Warren et al 1987). The initial step is reduction of the 5,6-olefinic bond of the uracil ring, which is catabolized by dihydrouracil dehydrogenase to form a 5,6-dihyropyrimidine. Subsequent hydrolytic reactions eventually result in the formation of carbon dioxide, ammonia and an alanine analog that is specific for each base. In contrast to FU and thymine, the 5-ethyluracil base derived from EDU does not appear to be good substrates for additional catabolic reactions (Joly et al 1991b), although EU binds tightly to dihydrouracil dehydrogenase (Naquib et al 1989).

The kinetics and biotransformation of EDU in both male Balb-C mice and male Spraque-Dawley rats were investigated as part of the thesis proposal. Mice received a 100 mg/kg dose of EDU via iv injection and oral administration, and rats were adminstrated a similar dose via jugular vein injection. Results from this study are summarized in Tables 13.3.6, 14.3.6 and in figures 8.3.6 and 9.3.6. EDU followed a two compartment kinetic model after a 100 mg/kg iv bolus injection in both male Balb-C mice and male Spraque Dawley rats. EDU also showed a very short distribution half-life in both species. However, the half-life of the terminal phase of EDU was shorter in rats than in mice, with half-lives of 18.5 and 24.1 min, respectively. The observation that EDU has a longer terminal half-life in mice, as well as a longer MRT (25.8 min) in mice compared to that in rats (11.0 min) indicates that the volume of distribution of EDU is different in mice and rats. However, the observed difference between blood clearance values of EDU in the two species was not significant ($P > 0.05$).

The extremely short distribution half-life for EDU shows that early blood concentrations are a crucial determinant of pharmacokinetic constants for EDU. However, the half-life of the terminal phase for EDU (24.1 min) is considerably longer than the reported value of 4.3 min for (E)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU) (Samuel et al 1986). It was also previously reported that EDU disappeared rapdily from plasma following po and ip administration in mice (Schinazi et al 1985) and after po and iv administration in rats (Hempel et al 1985, Kaul et al 1985, Buchele et al 1989). It was reported that 92% of the injected dose of [2-^{14}C]-EDU was excreted into urine during a 24 hr period post iv injection into rats (Kaul et al 1980). The rapid *in vivo* clearance of EDU from blood most likely corresponds to its extraction by other tissues such as liver, renal clearance and metabolism to EU and HEU.

The biotransformations of EDU in rats and mice were similar. EDU was rapidly metabolized to EU and subsequently hydroxylated to HEU (Figure 2.4.1).

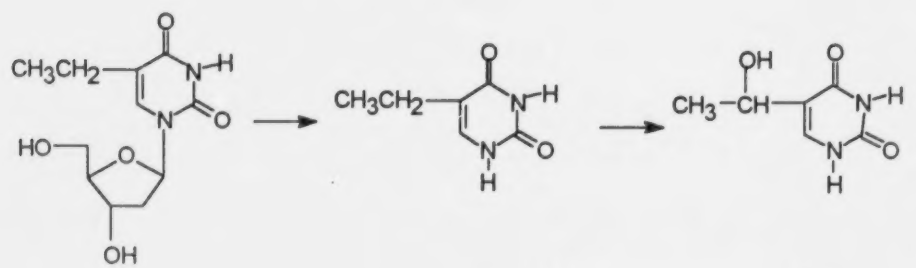


Figure 2.4.1. Metabolism of EDU in mice and rats.

Based on the observations that EU and HEU, but not HEDU, were detected in blood samples following administration of EDU, HEU is probably formed directly via hydroxylation of EU rather than via hydroxylation of EDU. However, biotransformation of EDU was less extensive in mice than in rats. The AUC for EU, a metabolite of EDU, after iv injection of EDU into mice was lower (249.2 ± 53.8 $\mu g.g^{-1}.min$) compared to that in rats (597.7 ± 354.1 $\mu g.g^{-1}.min$). This observation indicates that renal clearance of EDU is a more important pathway for elimination of EDU in mice than in rats. In fact, it was observed that an iv dose of ^{14}C-labelled EDU in mice was excreted predominately in urine (section 3.3.1). A similar observation was reported for [2-^{14}C]-CEDU, which bears a close structural similarity to EDU, where a higher percentage of an iv dose was excreted via the urine of mice relative to that of rats (Szinai et al 1991).

In contrast to the two compartment model after iv dosing, EDU followed a one compartment kinetic model in mice after a 100 mg/kg po dose. The concentrations of EDU in blood samples collected after po dosing were best fitted to a one compartment model with first order input (Figure 9.3.6). Based on the fact that EDU showed a very short distribution half-life following an iv bolus injection, it is believed that the distribution phase of EDU is superimposed on the absorption phase after po dosing. The maximum concentration (C_{max}) of EDU was 2.4 ± 0.2 μg/g at a T_{max} of 31.1 ± 1.2 min after dosing. However, the blood profile of EU, a metabolite of EDU, after a po dose is significantly different ($P < 0.05$) compared to that after an iv dose. This observation indicates that EDU undergoes catabolic degradation by gastrointestinal (GI) tract phosphorylases and/or similar hepatic enzymes before entering the circulation. These results and also the fact that the GI mucosa is rich in phosphorylases, suggest that some of the

EU present in blood samples collected after a po dose arises from the absorption of EU *per se* after cleavage of the glycoside bond in EDU. The degradation of other pyrimidine nucleosides, such as 5'-deoxy-5-fluorouridine and its prodrug trimethoxybenzoyl-5'-deoxy-5-fluorouridine by phosphorylases present in the GI tract has been reported (Ninomiya et al 1990).

EDU showed a 49% bioavailability in male Balb-C mice, which falls in the mid-range of reported values for other antiviral nucleosides. Ganciclovir, for example, has an oral bioavailability as low as 6%, whereas AZT has an oral bioavailability as high as 63% (Morse et al 1993, Klecker et al 1987). An oral bioavailability of 30-50% for EDU was estimated previously in rats (Hempel et al 1985). These numbers may suggest a "first pass" effect for EDU.

4.2. Biotransformation and antiviral activity of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridines

The BMEDU, BEEDU and CMEDU diastereomers were much more lipophilic than EDU. For example, the 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridines exhibited high octanol-water coefficients (P=0.38-2.5 range) relative to EDU (P=0.081) (Table 1.3.1). These compounds also show higher levels (11-22%) of binding to BSA than EDU (7%). The (5R,6R)-BMEDU and (5R,6R)-CMEDU diastereomers have the highest protein binding (%PB) to BSA (Table 1.3.1), which indicates the crucial role of configuration at C-5 and C-6 exert on the percent of protein binding of these compounds. However, BEEDU which also has a (5R,6R)-configuration showed a slightly lower %PB than that of (5R,6R)-CMEDU; its

P value was also lower than the BMEDU diastereomers and (5R,6R)-CMEDU, but substantially higher than (5S,6R)-CMEDU.

Although protein binding may play an important role in the biotransformation of a drug or transport through the BBB (Lin et al 1987), this occurs only for "highly" bound drugs (> 70%). Therefore it is very unlikely that the low level of protein binding of EDU, or its 5,6-dihydro prodrugs, plays an important role in their *in vivo* biotransformation.

The enhanced lipophilicity of these 5,6-dihydro compounds may enable them to enter cells more readily by diffusion. The high blood concentration of the CMEDU diastereomers, in conjunction with their enhanced lipophilicity, suggests that these compounds should provide a higher concentration in the brain than EDU. This postulate is based on the observation that brain capillary permeability is often related to the octanol-water partition coefficients and molecular weights of the compounds. Increasing the lipophilicity of compounds with a molecular weight of less than 400 has been reported to improve brain permeability (Levin 1980).

The *in vitro* antiviral test data for EDU, BMEDUs and CMEDUs against HSV-1 and HSV-2 are summarized in Table 1.4.1. (5R,6R)-BMEDU, and (5S,6S)-BMEDU diastereomers exhibited equipotent *in vitro* antiviral activity to that of the reference drug (EDU) against both HSV-1 and HSV-2. In contrast, the (5R,6R)-CMEDU, and (5S,6R)-CMEDU diastereomers were both inactive against HSV-1 and HSV-2. These results suggest that the (5R,6R)-BMEDU, and (5S,6S)-BMEDU diastereomers, which are much more lipophilic (log P = 0.28-0.40) than EDU (log P = -1.09), would be more useful for the treatment of HSE than EDU. In the HCMV antiviral assay, (5S,6S)-BMEDU exhibited 1/10 of the potency of ganciclovir, whereas (5S,6R)-CMEDU was approximately equipotent to ganciclovir. These latter

results indicate that the nature of the halogen atom at C-5 and the configuration at C-5 and/or C-6 positions, are determinants of HSV-1 and HSV-2 antiviral activities: [(5S,6S)-BMEDU and (5R,6R)-BMEDU >> (5R,6R)-CMEDU and (5S,6R)-CMEDU], and for HCMV, [(5S,6S)-BMEDU >> (5R,6R)-BMEDU; (5S,6R)-CMEDU >> (5R,6R)-CMEDU]. The fact that (5S,6R)-CMEDU was equipotent to ganciclovir against HCMV suggests it could serve as a useful lead-compound for development of an improved anti-HCMV drug which is urgently required for antiviral chemotherapy.

Table 1.4.1. *In vitro* antiviral activity of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridines.

	EC_{50} (μM)[a]		
Compound	HSV-1 (E-377)[b]	HSV-2 (MS)[b]	HCMV (AD169)[b]
(5R,6R)-BMEDU	0.29	0.53	>50
(5S,6S)-BMEDU	0.67	0.13	0.28
(5R,6R)-CMEDU	>50	>50	>50
(5S,6R)-CMEDU	>50	>50	0.04
EDU	0.29	0.38	0.06
Acyclovir	0.03	0.03	ND[c]
Ganciclovir	ND	ND	<0.03

[a]The drug concentration (μM) required to reduce the viral cytopatic effect (CPE) in infected monolayers to 50% of untreated uninfected controls.
[b]The strain of virus which was used for antiviral testing.
[c]Not determined.

Biotransformation of (5R,6R)-BMEDU, (5S,6S)-BMEDU, (5R,6R)-CMEDU and (5S,6R)-CMEDU, relative to that of EDU, were investigated by

injection of a 0.7 mmol/kg dose of the test compound into male Spraque-Dawley rats via a jugular catheter. Results from this study are summarized in Table 2.3.2 and figures 1.3.2 and 2.3.2.

Since EDU undergoes rapid catabolism to the inactive EU in the presence of pyrimidine phosphorylases, 5,6-dihydro prodrugs were designed in order to prevent or reduce this degredation. There is precedent for this concept, since it has been reported that 5-bromo-5-fluoro-6-methoxy-5,6-dihydro-2'-deoxyuridine was not cleaved by either *E.coli* nucleosidase or a nucleoside phosphorylase prepared from Ehrlich ascites cells which readily degraded FUDR to FU (Duschinsky et al 1967). The results from experiments with 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridines show that these 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridines should be useful prodrugs to EDU, with a longer blood residence time. These 5,6-dihydro prodrugs could undergo *in vivo* regeneration of the 5,6-olefinic bond to produce the active EDU. It is expected that this rate of the regeneration should be dependent upon the nature of the C-5 halogen substituent.

Separation of the parent compound, base and hydroxy metabolites by HPLC usually requires specific conditions (Joly et al 1991a). Since there are significant differences in the solubility and partition characteristics of the 5,6-dihydro compounds used in this study, relative to EDU, it was necessary to develop a gradient elution HPLC method to separate the parent 5,6-dihydro compound and its metabolites. The HPLC conditions described in section 3.2 provided good resolution of the 5,6-dihydro compounds, EDU, EU and their hydroxylated metabolites (Figure 3.4.2).

A putative metabolic pathway for the biotransformations observed for the 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds

investigated is presented in figure 4.4.2. The formation of HEDU, as a metabolite of EDU in urine or blood, has not previously been reported. In contrast, in this study HEDU was detected, but not quantitated, in the extract of blood samples collected following injection of the bromo compounds (5R,6R)-BMEDU and (5S,6S)-BMEDU (Figure 3.4.2). Since the quantity of hydroxyl metabolite present in blood samples following injection of the chloro compounds (5R,6R)-CMEDU and (5S,6R)-CMEDU was very low, it is likely that HEDU arises directly from the parent 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridine (Figure 4.4.2, pathway A). Although EDU and EU were present in significant levels in blood samples following injection of the two bromo BMEDU diastereomers, the concentration of these metabolites was much lower for the two chloro CMEDU diastereomers. These results indicate that the chloro CMEDU diastereomers, especially (5R,6R)-CMEDU, are less susceptible to *in vivo* metabolism. Clearly, the *in vivo* stability of these 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds is dependent upon the nature of the halogen substituent, with the chloro being more stable than the bromo analogs. The higher concentrations of the CMEDU diastereomers present in blood indicate that they are more stable, are oxidized very slowly to HEDU, are more stable to pyrimidine phosphorylase, and are converted slowly to EDU.

(5R,6R)-CMEDU showed significantly different characteristics than that of (5S,6R)-CMEDU (Table 2.3.2 and 1.3.1). For example, (5R,6R)-CMEDU showed a substantially higher *in vivo* stability and therefore lower clearance and longer half-life than (5S,6R)-CMEDU. These observations clearly show that in addition to nature of C-5 substituents, the configuration of substitutents may also play a crucial role in *in vivo* behavior of the 5,6-dihydro prodrugs.

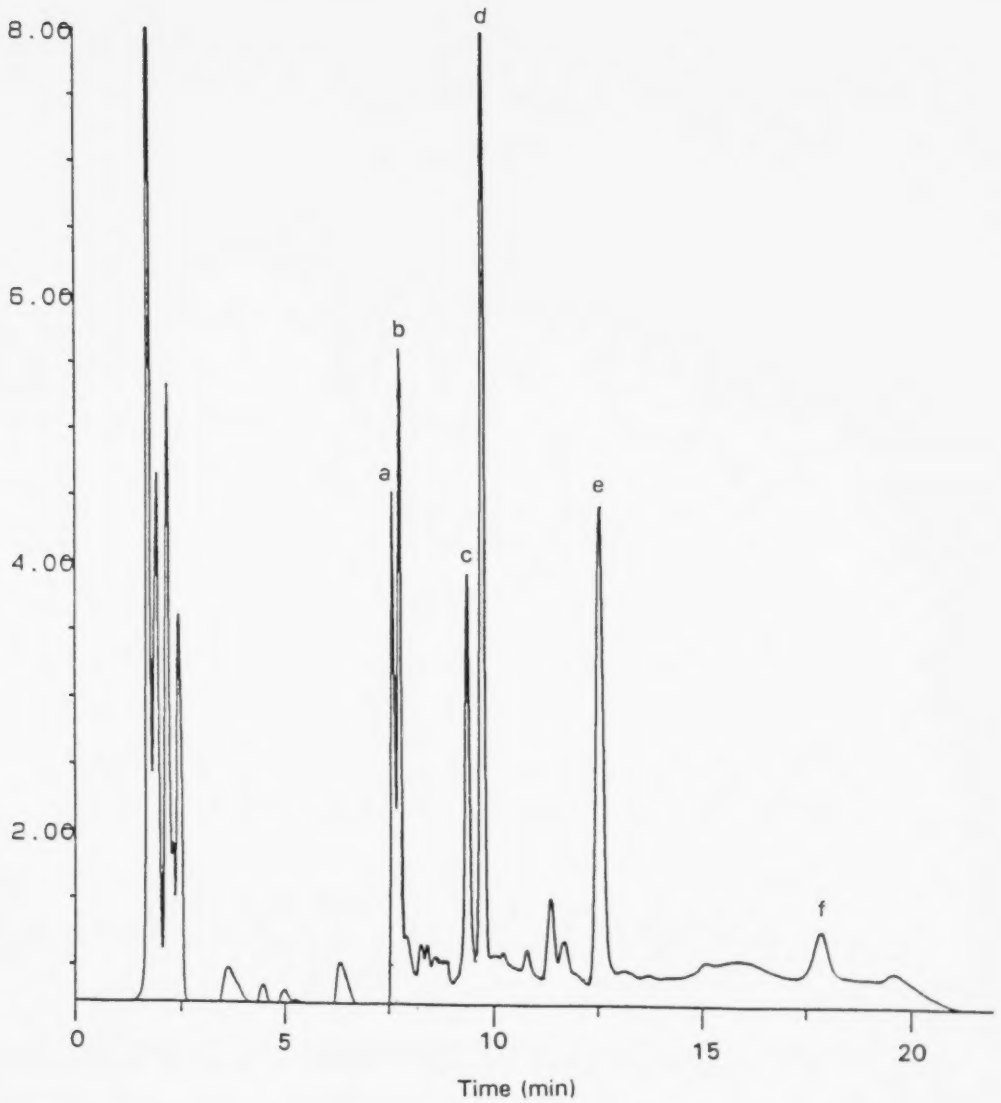


Figure 3.4.2. HPLC analysis of rat-blood extract after injection of (5R,6R)-BEMDU. Retention time (min): HEDU (a,7.56), HEU (b,7.75), EU (c,9.35), EDU (d,9.73), Internal Standard (e,12.54) and (5R,6R)-BMEDU (f,17.88).

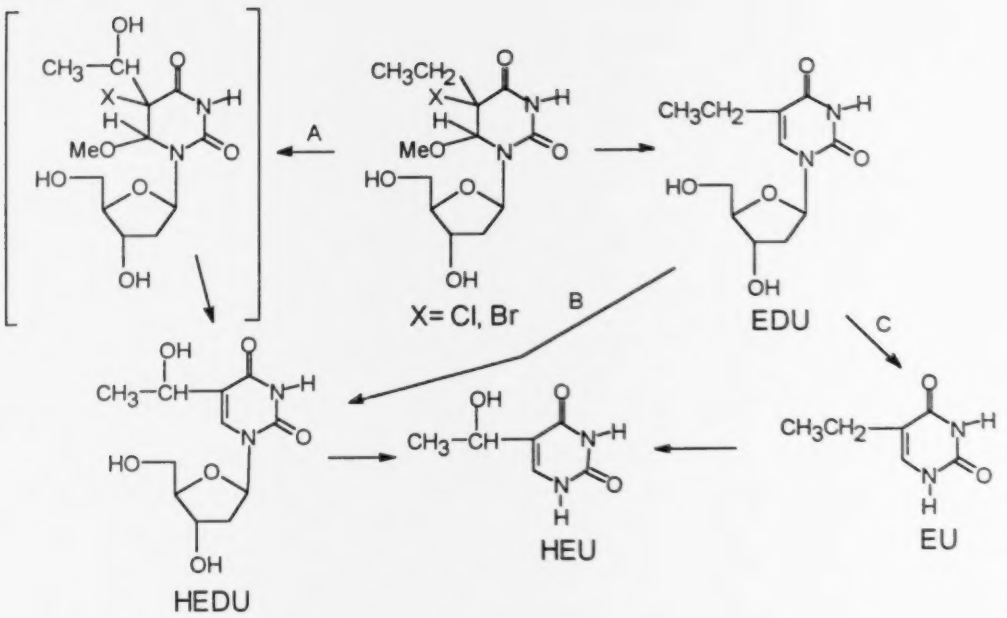


Figure 4.4.2. Putative metabolic transformation of 5-ethyl-5-halo-6-methoxy-2'-deoxyuridines *in vivo*. Path A, formation of HEDU and HEU via a 5-halo-6-methoxy intermediate; Path B, formation of HEDU and HEU via EDU (presence of HEDU in blood after injection of EDU into rats was neither detected in these experiments nor reported previously); and Path C, formation of HEU via EU.

Although regeneration of the 5,6-olefinic bond is an important step in the biotransformation of these 5,6-dihydro prodrugs, there is limited information available regarding the actual mechanism involved for the regeneration of the 5,6-olefinic bond *in vivo*. Most of the literature data are related to elimination of halogen atom in 5-halo-2'-deoxyuridines. In general, it is believed that dehalogenation of 5-halo-2'-deoxyuridines is catalyzed by nucleophilic groups. It was reported that dehalogenation of both 5-bromo-5,6-dihydrouracil and 5-bromo-6-methoxy-5,6-dihydrouracil *in vivo* proceeds via an NADP-linked, dihydrouracil dehydrogenase-catalyzed reduction of the 5,6-double bond of the halouracil followed by a nonenzymatic E_2 elimination of HX from the halodihydrouracil intermediate to yield uracil (Kriss et al 1962, Hampton et al 1961). It was also reported that bisulfite buffers dehalogenate 5-bromouracil (BrU) via a multistep reaction pathway which involves SO_3^{2-} attack on C-6 of BrU to yield the enolate anion of 5-bromo-5,6-dihydrouracil-6-sulfonate. General acid catalyzed protonation of this anion and finally SO_3^{2-} attack on the anion yields both uracil and 5,6-dihydrouracil-6-sulfonate as products (Sedor et al 1975). It was also reported that hydrosulfide groups can attack at the bromine atom in 5-bromo-5-fluoro-6-methoxy-5,6-dihydrouracil, with elimination of bromide from the molecule to give FU (Robins et al 1976). Rork et al reported that the attack of sulfite ion on the molecule is the rate-determining step in the dehalogenation process (Rork et al 1975).

Based on these results, and the results of our study, plausible mechanisms for the conversion of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridines to EDU are shown in figure 5.4.2. It is likely that dehalogenation and elimination to generate EDU occurs by reaction with glutathione (GSH), or related tissue nucleophiles such as cysteine. It has

been shown that *in vitro* incubation of 5-bromo-5-fluoro-6-methoxy-5,6-dihydro-2'-deoxyuridine diastereomers with GSH regenerates the 5,6-olefinic bond to give FUDR (Duschinsky et al 1967). The reaction with GSH (RSH) could occur by two mechanisms.

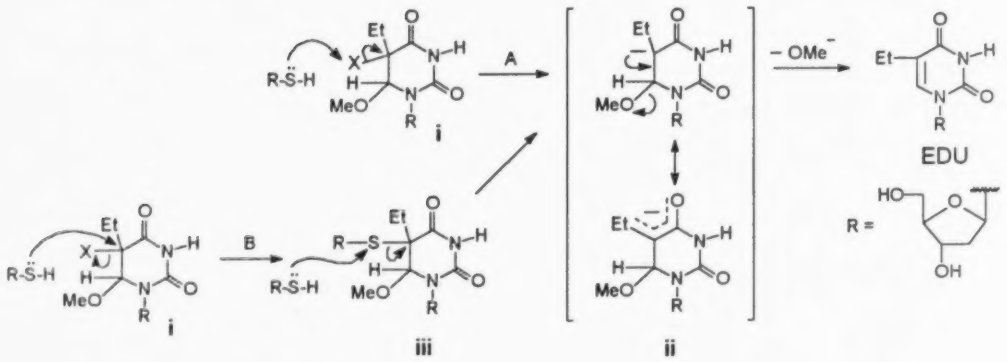


Figure 5.4.2. Proposed mechanisms for the *in vivo* conversion of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridines to 5-ethyl-2'-deoxyuridine. Path A, elimination under the influence of a nucleophile; and Path B, nucleophilic substitution of X by R-S-H, followed by elimination of R-S- under the influence of a second nucleophile.

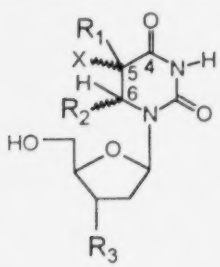
Elimination of the C-5 halo substituent through nucleophilic attack by RSH would give the carbanion or enolate anion (ii) (Figure 5.4.2, pathway A). Alternatively, a S_N2 displacement of halogen by RSH to give (iii)

(Figure 5.4.2, pathway B) and a subsequent reaction with GSH could also yield the carbanion (ii). Elimination of methoxide anion from the carbanion intermediate (ii) would regenerate the 5,6-olefinic bond to afford EDU.

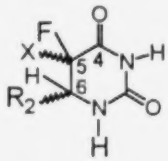
However, further experiments with other 5,6-dihydro prodrugs of EDU such as BEEDU, revealed that there might be another mechanism(s) involved in regeneration of the 5,6-olefinic bond. In contrast to what was expected, no regeneration of the 5,6-olefinic bond was observed upon incubation of BEEDU with GSH. However, this prodrug showed significant conversion to EDU upon incubation with rat blood and also rat brain homogenate (section 3.4). Regeneration of the 5,6-olefinic bond of BEEDU was less pronounced upon incubation with rat plasma.

The results of the *in vitro* incubation with GSH in this study, and those reported previously, suggest that the mechanism(s) for regeneration of the 5,6-olefinic bond present in the uracil ring require further explanation. Data summarizing the regeneration of the 5,6-olefinic bond in the uracil ring of the various 5,6-dihydro derivatives of some pyrimidine nucleosides and uracil are summarized in Table 2.4.2. Several conclusions can be drawn from these results. First, these results show that all 5,6-dihydro derivatives with Cl at C-5 (A12-A16), regardless of nature of the other alkyl substituent at C-5, did not undergo regeneration of the 5,6-olefinic bond upon *in vitro* incubation with GSH. Second, all of the 5-bromo-6-alkoxy-5,6-dihydro compounds (A2-A9, D1-D2, B1-B3, C1-C2), with the exception of compound A1, regenerated the 5,6-olefinic bond upon incubation with GSH. The extent of 5,6-double bond regeneration varied over a 10-100% range.

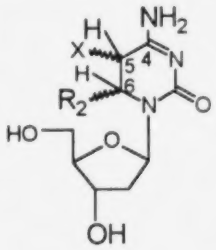
Table 2.4.2. Regeneration of the 5,6-olefinic bond present in compounds A-D upon *in vitro* incubation with GSH.



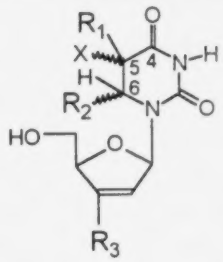
A



B



C



D

Compound	R_1	X	R_2	R_3	otation and/or Configuration	% Regenerated 5,6-Olefinic Parent
A1	Et	Br	OEt	OH	(+)-(5R,6R)	0[a]
A2	F	Br	OEt	OH	(+)	18[b]
A3	Et	Br	OMe	OH	(+)-(5R,6R)	40[a]
A4	Et	Br	OMe	OH	(-)-(5S,6S)	10[a]
A5	F	Br	OMe	OH	(-)	20[b]
A6	F	Br	OtBu	OH	ND[c]	10[b]
A7	F	Br	OMe	OH	(+)	23[b]
A8	Me	Br	OMe	N_3	(+)-(5R,6R)	98[d]
A9	Me	Br	OMe	N_3	(-)-(5S,6S)	60[d]
D1	Me	Br	OMe	H	(-)-(5S,6S)	50[d]
D2	Me	Br	OMe	H	(+)-(5R,6R)	85[d]
B1	-	Br	OMe	-	(±)	37[b]
B2	-	Br	OEt	-	(±)	32[b]
B3	-	Br	OtBu	-	(±)	32[b]
C1	-	Br	OEt	-	(±)	100[b]
C2	-	Br	OMe	-	(±)	100[b]
A12	Et	Cl	OMe	OH	(+)-(5R,6R)	0[a]
A13	Et	Cl	OMe	OH	(+)-(5S,6R)	0[a]
A14	F	Cl	OMe	OH	ND[c]	0[b]
A15	Me	Cl	OMe	N_3	(+)-(5R,6R)	0[d]
A16	F	Cl	OtBu	OH	(+)	0[b]

[a]After a 24 hr incubation with GSH.
[b]Data from Duschinsky et al 1967. Data reported are percent conversion after a 36 hr incubation with GSH.
[c]Not determined.
[d]Data from Wang et al. Data reported are the percent conversion after a 30 min incubation with GSH.

It is clear that the configuration of the 5,6-dihydro compound plays a crucial role in the *in vitro* regeneration of 5,6-olefinic bond upon incubation with GSH. The nature of the alkyl group at C-6 in the 5-bromo-6-alkoxy-5,6-dihydro derivatives of pyrimidine nucleosides is also a determinant of the extent to which regeneration of the 5,6-double bond occurs. In contrast to compounds possessing a C-6 methoxy group, the extent of conversion of 5,6-dihydro compounds that have either C-6 OEt or t-BuO substituents was not higher than 32%. Third, the carbonyl moiety at C-4 also plays a crucial role in regeneration of the 5,6-olefinic bond, since replacement of the carbonyl group (class A, Table 2.4.2) with NH_2 (class C, Table 2.4.2) drastically increased the extent of conversion of the 5,6-dihydro derivatives to the parent nucleoside (compounds C1 and C2). Based on these observations, it appears that *in vitro* regeneration of the 5,6-olefinic bond upon incubation with GSH may not be a good model for prediction of the *in vivo* biotransformation of 5,6-dihydro derivatives to the parent 5,6-olefinic nucleoside. It has been reported that compounds A12, A13 and A15 are converted to their 5,6-olefinic parent compound following iv injection into mice and/or rats or *in vitro* incubation with blood (Wang et al 1994, Cheraghali et al 1994). Although A1 is stable during *in vitro* incubation with GSH, it showed a very short half-life *in vivo* and upon *in vitro* incubation with rats whole blood, but not with plasma.

4.3. Biodistribution and brain uptake of [4-^{14}C]-labelled (5R,6R)-BMEDU and (5S,6S)-BMEDU

The 5,6-dihydro compounds are more lipophilic than EDU, which should enhance their ability to cross the blood-brain barrier (BBB) and enter the brain. High brain uptake of an antiviral drug, which provides a

therapeutically effective brain concentration, is essential for the treatment of HSV infections of CNS such as HSE. In order to measure brain uptake of BMEDU diastereomers of EDU, [4-^{14}C]-labelled (5R,6R)-BMEDU and (5S,6S)-BMEDU were synthesized and their biodistributions, relative to that of [4-^{14}C]-EDU, were determined in mice. Balb-C mice received 126 kBq (3.4 μCi) of radiolabelled compound by iv injection. Each test compound was mixed with 0.2 mmol/kg of non-radioactive test compound. The results of this study are summarized in Tables 4.3.3.1 and 5.3.3.1 and also figure 4.3.3.1.

The radioactivity present in liver samples after injection of [4-^{14}C]-(5R,6R)-BMEDU and [4-^{14}C]-(5S,6S)-BMEDU was higher than that after injection of [4-^{14}C]-EDU, which indicates that these 5,6-dihydro derivatives undergo a higher hepatic extraction than EDU. However, radioactivity clearance from blood after injection of [4-^{14}C]-EDU was much faster than clearance from blood after injection of [4-^{14}C]-(5R,6R)-BMEDU or [4-^{14}C]-(5S,6S)-BMEDU. These differences are more substantial at early times post injection of BMEDU prodrugs and EDU. The overall radioactivity levels in brain samples after injection of [4-^{14}C]-(5R,6R)-BMEDU or [4-^{14}C]-(5S,6S)-BMEDU was not significantly ($P > 0.05$) greater than after [4-^{14}C]-EDU injection.

It was shown that the 5,6-dihydro BMEDU prodrugs also follow a metabolic pathway similar to that of EDU (section 4.2). Therefore, formation of EU (which has substantially longer half-life than EDU) and other metabolites following injection of [4-^{14}C]-labelled EDU or these 5,6-dihydro derivatives is thought to be responsible for the slow clearance of radioactivity from blood.

Presumably the high concentration of GSH in brain (Mcliwain et al 1985) might facilitate regeneration of the 5,6-olefinic bond in brain, which would result in production of the highly hydrophilic antiviral drug EDU. Due to its low lipophilicity, EDU will be trapped in brain long enough to exert its antiviral effects in virus-infected areas of the CNS. There are, however, competing kinetic processes. On one hand, the lipophilic and highly diffusable prodrug enters and exits brain tissue in response to concentration, but its concentration in brain tissue is also limited by rapid *in vivo* regeneration of the 5,6-double bond in peripheral tissues. The process of regeneration of the 5,6-double bond in peripheral tissues will result in production of highly hydrophilic drug (EDU) which is incapable of diffusing back across the BBB in significant levels. It appears that either diffusion of these BMEDU diastereomers (due to increased lipophilicity) across the BBB is not sufficient to provide an initial high concentration of the 5,6-dihydro prodrug in brain, or that regeneration of the 5,6-olefinic bond in brain is slow relative to the reversible diffusion across the BBB. Once the 5,6-olefinic bond is regenerated, however, metabolism and excretion kinetics of the prodrugs become those of EDU, and no further prodrug advantage remains.

The results of this study of the 5-bromo-6-methoxy-5,6-dihydro derivatives of EDU indicate that some molecular redesign may be necessary. Increased lipophilicity and/or adjustment of the regeneration rate and the regeneration mechanism for these 5,6-dihydro prodrugs may be necessary in order to achieve a significantly higher, therapeutic concentration of active drug in the brain.

4.4. *In vitro* and *in vivo* evaluation of BEEDU

Based on results from studies for BMEDU and CMEDU diastereomers, other 5,6-dihydro prodrugs of EDU were designed in order to obtain improved pharmacokinetics and also brain uptake relative to that of EDU. Since CMEDU diastereomers did not show antiviral activity against HSV and also did not show good conversion to EDU following iv injection into rats, the 5-bromo-6-ethoxy-5,6-dihydro derivative of EDU was selected as a new prodrug. However, the anti-HCMV effect of (5R,6R)-CMEDU is the subject of further ongoing investigation. BEEDU was expected to be a more lipophilic prodrug and due to its longer chain substituent on C-6 (ethoxy vs methoxy) to show more readily conversion *in vivo* to parent drug EDU.

In contrast to what was expected, BEEDU did not show a higher lipophilicity than the BMEDU diastereomers (Table 1.3.1). In fact its lipophilicity was substantially lower than that of (5S,6S)-BMEDU and the homolog prodrug, (5R,6R)-BMEDU. One explanation for this result might be due to fact that P values for the BMEDU diastereomers and CMEDU diastereomers were measured using slightly different conditions (see Table 1.3.1). However, BEEDU showed a substantially higher protein binding to BSA than (5S,6S)-BMEDU and it showed a similar %PB to that of (5R,6R)-BMEDU (Table 1.3.1).

BEEDU did not undergo conversion to EDU in normal saline or upon incubation with 2 molar equivalents of GSH at 37°C for 36 hr, but it was converted to EDU after a 2 hour incubation with rat plasma (8%), rat brain homogenate (16%) and rat whole blood (53%). Conversion of BEEDU to EDU upon incubation with brain homogenate clearly shows that brain is capable of activating BEEDU, and therefore trapping EDU as a result of this

activation process. However, significant differences in the rate of conversion of BEEDU upon incubation with whole blood and plasma also shows that blood cells could play an important role in conversion of BEEDU to EDU.

Biotransformation of BEEDU, relative to that of EDU, was investigated in male Sprague-Dawley rats having an implanted jugular catheter. Animals received 0.55 mmol/kg of either BEEDU or EDU. BEEDU was rapidly cleared from blood after iv injection into rats, and no BEEDU was detected in blood samples taken 18 min post injection. However, as expected from the *in vitro* studies, BEEDU was converted to EDU, providing high concentrations of EDU in blood. The AUC(0 → last sample) of EDU after injection of BEEDU, was significantly higher (4.25 μmol.hr.ml^{-1}) than after injection of EDU (1.83 μmol.hr.ml^{-1}). In fact, the concentration of EDU in blood samples taken 3 and 4 hr post injection of the prodrug were much higher than after injection of EDU (Figure 7.3.5). The AUC(0 → last sample) of EU, a metabolite of EDU, was also higher after injection of BEEDU compared to that after injection of EDU (Table 12.3.5). These observations indicate that although the concentration of prodrug in blood samples taken >18 min post injection was below the HPLC detection limit (0.4 μg/mL), the prodrug is present in other tissues and acts as a slow releaser of EDU into the vascular compartment.

A plausible metabolic pathway for the biotransformation of BEEDU is presented in figure 6.4.4. After conversion to EDU, EU and HEU are produced as secondary metabolites of BEEDU. Although HEDU was detected as a metabolite of 5-halo-5-ethyl-6-methoxy-5,6-dihydro derivatives of EDU after injection into rats (section 3.2), HEDU was not detected in blood samples taken from rats receiving BEEDU. It was

previously postulated (section 4.2) that HEDU most likely arises from oxidative attack at C-1 of the 5-ethyl substituent of 5-halo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridines. This difference in the metabolic pathways of BEEDU and the 5-halo-5-ethyl-6-methoxy-5,6-dihydro derivatives of EDU may arise from the fact that *in vivo* conversion of BEEDU to EDU in blood is much faster than that of 5-halo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridines.

BEEDU exhibited a higher octanol/water coefficient (P = 1.1) than EDU (P = 0.08). The enhanced lipophilicity of BEEDU should enable it to enter cells and cross the BBB more readily than EDU, by diffusion. This postulate is substantiated by the [4-^{14}C]-BEEDU biodistribution study in Balb-C mice. Radioactivity levels present in brain samples taken after

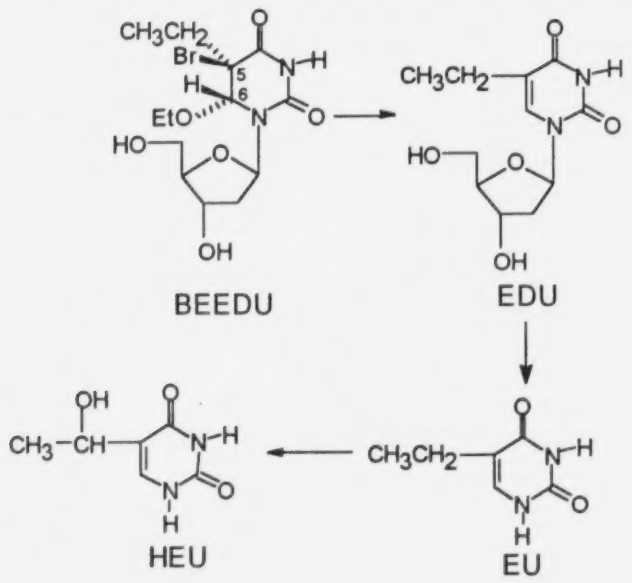


Figure 6.4.4. Proposed metabolic pathway for BEEDU.

injection of 126 kBq (3.4 μCi) [4-^{14}C]-BEEDU, mixed with 0.2 mmol/kg of non-radioactive BEEDU, into the tail vein of mice were significantly higher ($P < 0.05$) than those for [4-^{14}C]-EDU (Table 6.3.3.1 and 3.3.3.1).

The substantially higher levels of radioactivity in muscle after injection of [4-^{14}C]-BEEDU relative to those after injection of [4-^{14}C]-EDU, further indicate that the 5,6-dihydro prodrug can readily penetrate other tissues. Since *in vivo* and *in vitro* studies showed that BEEDU is rapidly converted to EDU, and although BEEDU will diffuse out of the cell at the same rate at which it diffuses into the cell ($k_1=k_{-1}$, Figure 7.4.4), its very rapid conversion will produce a significantly more hydrophilic compound (EDU) which may diffuse out of cell much more slowly than the prodrug ($k_3 << k_{-1}$). This alone will cause elevated levels of EDU in cells. Moreover, further conversion of EDU to its highly hydrophilic monophosphate derivative (P-EDU) by HSV thymidine kinase in virus-infected cells will result in complete trapping of the active form of drug in cells. Since the rate of conversion of BEEDU to EDU (k_2) is very fast, the rate of phosphorylation of EDU in HSV infected cells (k_4) would be the only rate-determining step in the complete trapping of BEEDU within virus infected cells. The higher levels of radioactivity present in liver samples after injection of [4-^{14}C]-BEEDU, relative to that of [4-^{14}C]-EDU, indicate that this 5,6-dihydro prodrug undergoes a higher hepatic partition than EDU. However, the observations that clearance of radioactivity from blood and excretion of radioactivity into urine after injection of [4-^{14}C]-BEEDU were much slower than that of [4-^{14}C]-EDU, indicate that the prodrug is exerting a marked depot effect.

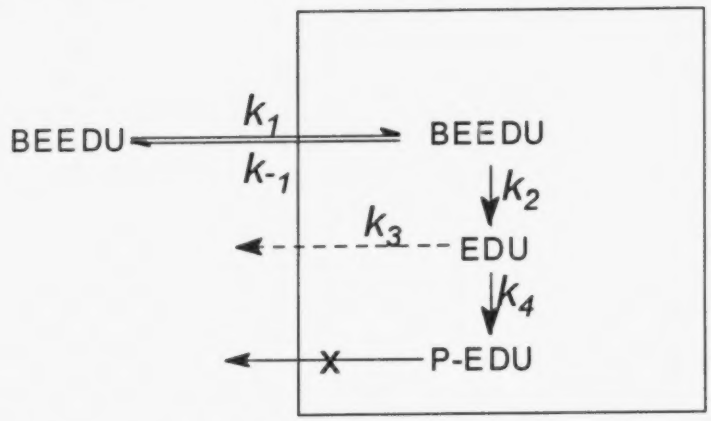


Figure 7.4.4. Proposed activation and trapping pathway for BEEDU in virus infected cells. P-EDU is monophosphate derivative of EDU.

The differences between excretion of radioactivity and higher radioactivity levels in muscle samples taken after injection of [4-^{14}C]-BEEDU compared to those of [4-^{14}C]-EDU indicate that prodrug may stay in tissues such as muscle and act as a slow releaser of EDU.

It was previously observed that 5-bromo-6-methoxy-5,6-dihydro derivatives of EDU did not provide a significantly higher brain concentration relative to EDU (section 3.3.1) and that they did undergo a slower *in vivo* conversion to EDU (section 3.2) relative to the rapid conversion of BEEDU observed in this study. However, the current observation that BEEDU provided a significantly higher brain concentration indicates that in contrast to its faster conversion into EDU, the increased lipophilicity of BEEDU is sufficient to provide an initial higher concentration of prodrug in brain prior to the regeneration of 5,6-olefinic bond in peripheral tissues. Once the 5,6-olefinic bond is regenerated, antiviral activity, metabolism and excretion

kinetics in brain and all other tissues would be the same for the prodrug as for EDU.

It was postulated that possible diffusion of these highly lipophilic prodrugs following bolus injection into the relatively small tail vein of mice may have reduced the amount of injected radiolabelled test compound in the circulation, at least in the early post injection periods. This may have finally resulted in reduction of radioactivity levels (% injected dose) in brain samples. In order to investigate the effect of route of injection, [4-^{14}C]-BEEDU was injected into the jugular vein which is relatively a large vein. The results of this study are presented in figure 8.4.4.

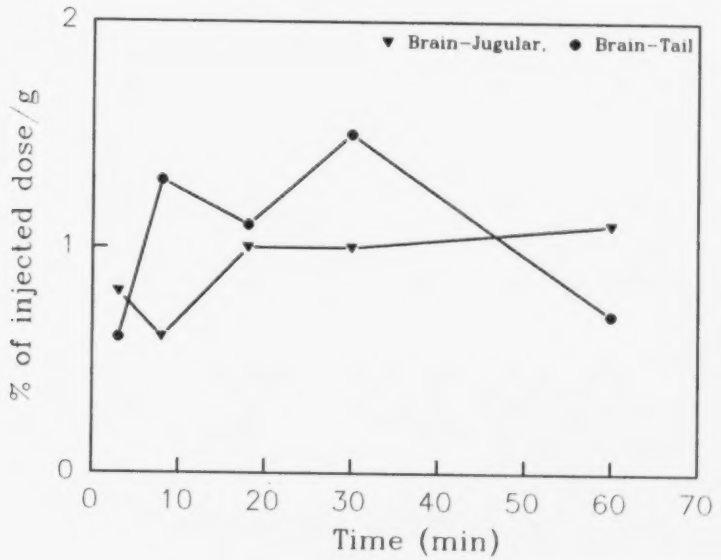


Figure 8.4.4. Radioactivity present in brain samples after injection of [4-^{14}C]-BEEDU via the tail vein and jugular vein. Data are the means of three separate experiments.

However, no significant increase ($P > 0.05$) was observed in the quantity of radioactivity present in brain samples taken after injection of [4-^{14}C]-BEEDU into the jugular vein. The results of the *in vitro* and *in vivo* comparative studies for BEEDU and EDU described in this study clearly indicate that this 5,6-dihydro compound could serve as a useful brain-targeted prodrug for EDU.

4.5. *In vitro* and *in vivo* evaluation of double prodrugs of EDU

The results of the studies with BEEDU and BMEDU diastereomers prompted us to design a candidate double prodrug to EDU in order to further improve the delivery characteristics and therapeutic efficacy of EDU and also examine the effect of a further increase in prodrug lipophilicity on brain uptake, biodistribution and biotransformation of the prodrug and EDU. The double prodrug was designed to undergo activation via a cascade mechanism. The initial step of the activation mechanism was designed to proceed via an enzymatic ester hydrolysis that would be followed by non-enzymatic regeneration of the 5,6-olefinic bond. A valeryl moiety was selected for esterification of the 5'-hydroxyl group since it was reported that the 5'-O-valeryl ester of IDU has a very short half-life upon incubation with rat plasma and it is more lipophilic than other 5'-O-aliphatic esters of IDU (Gosch et al 1991). It was also reported that VEDU exhibited similar antiviral activity to EDU, presumably due to its rapid conversion to EDU (Keppler et al 1986). VBEEDU and DVBEEDU were synthesized by esterification of BEEDU using valeryl chloride. Relative to EDU and other 5,6-dihydro prodrugs of EDU, VBEEDU and DVBEEDU were found to be much more lipophilic. VBEEDU has a P value of 11.5 which is 10-fold higher

than that of BEEDU (Table 1.3.1). It was not possible to measure the P value for DVBEEDU, using the HPLC method, since no DVBEEDU was detected in buffer phosphate phase, during partition coefficient determination.

VBEEDU undergoes rapid enzymatic hydrolysis to BEEDU upon incubation with porcine liver esterase at 37°C, since more than 95% of VBEEDU was hydrolyzed after an 8 min incubation. This rapid hydrolysis of the valeryl ester moiety of VBEEDU upon incubation with porcine liver esterase, is in agreement with the reported value for the hydrolysis of the 5'-O-valeryl ester of IDU (Ghosh et al 1991). No chemical hydrolysis of VBEEDU was observed using a control experiment not containing the enzyme. This observation indicates that VBEEDU is chemically stable in aqueous solution.

There is precedence that increasing the lipophilicity of compounds with a molecular weight of about 400 enhances their brain permeability (Levin 1980, Hardebo et al 1979). In the absence of an active transport system, the enhanced lipophilicity of a prodrug should facilitate the entry into cells and the ability to cross the BBB more readily by diffusion than EDU. Although VBEEDU and BEEDU may diffuse out of the cell at the same rate at which they diffuse into the cell, rapid regeneration of the 5,6-olefinic bond and enzymatic hydrolysis of the 5'-O-ester moiety would afford the more polar, less lipophilic EDU that should be retained in the cell to a greater extent. The subsequent conversion of EDU to its highly hydrophilic 5'-O-monophosphate derivative by HSV encoded-thymidine kinase in virus-infected cells would result in complete trapping.

It was previously observed that BEEDU provided a significantly higher ($P < 0.05$) brain uptake after iv injection of the [4-^{14}C]-radiolabelled

compound into mice, compared to EDU. Therefore it was expected that VBEEDU, which has a P value one-log order larger than BEEDU, would provide a higher brain concentration. Esterification of one hydroxyl group was expected to dramatically enhance brain uptake, since it was previously reported that the presence of a single hydroxyl group decreased BBB permeability 10-fold (Partridge 1979). An effective antiviral compound for the treatment of cephalic infections such as HSE must cross the BBB effectively and localize in virus infected tissues to provide a clinically effective drug concentration.

In order to initiate the *in vitro* and *in vivo* studies described, an efficient quantitative HPLC analytical procedure was developed to separate VBEEDU and its metabolites in blood samples. A HPLC procedure had been developed (section 3.3) which provided a good separation of 5-halo-6-methoxy-5,6-dihydro prodrugs of EDU, EDU and its metabolites. However, the HPLC separation of VBEEDU and its metabolites required a more complex gradient of acetonitrile and water, in combination with a flow rate gradient. This HPLC procedure , which is summarized in Table 1.2.13, provided an excellent separation of VBEEDU and its metabolites in blood (Figure 9.4.5). The quantitative HPLC data were used to calculate the pharmacokinetic parameters and elucidate the cascade reactions responsible for activation of VBEEDU *in vivo*.

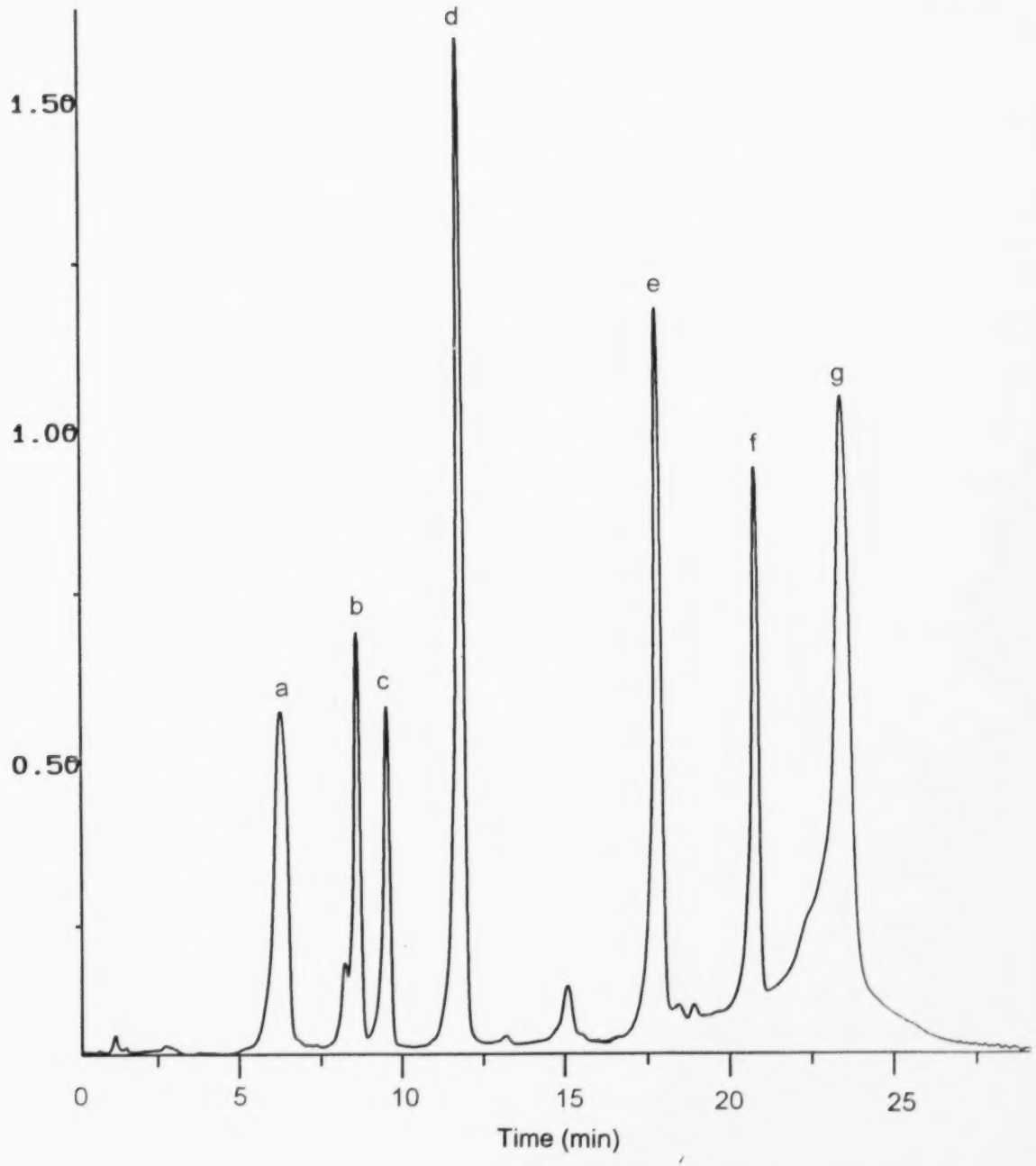


Figure 9.4.5. HPLC separation of a mixture of HEU (a), EU (b), EDU (c), Internal Standard (Caffeine, d), BEEDU (e), VEDU (f) and VBEEDU(g).

In a comparative study, the biotransformations of EDU, BEEDU and VBEEDU were investigated by injection of a 0.4 mmol/kg dose of the test compound into the lateral tail vein of male Balb-C mice. The mean blood concentration-time profiles for these studies are shown in figure 10.3.8. BEEDU showed a very short half-life following iv injection, since its presence could not be detected in blood samples collected 10 min post injection, whereas the concentration of EDU in blood was high. The rapid clearance of BEEDU from blood is therefore due, at least in part, to its conversion to EDU.

VBEEDU provided a consistent, but lower than that of BEEDU, concentration of EDU in blood following iv injection into mice. VBEEDU and VEDU were detected in blood samples collected up to 35 min post injection. This observation indicates that the $t_{1/2}$ for VBEEDU was longer than might be predicted based on the *in vitro* incubation experiments. In contrast to BEEDU, which could not be detected at 10 min post injection time, VEDU was detected in blood samples up to 35 min post injection. A plausible mechanism for activation of VBEEDU, based on these observations, is shown in figure 10.4.5. In this biotransformation pathway, VBEEDU is converted into BEEDU and VEDU simultaneously (k_1, k_2). BEEDU and VEDU are subsequently converted to EDU by regeneration of the 5,6-olefinic bond (BEEDU) and hydrolysis of the 5'-O-valeryl ester (VEDU), respectively (k_3, k_4). However, the results from this study, and a previous study of BEEDU in rats, show that *in vivo* regeneration of 5,6-olefinic bond in BEEDU occurs at a much faster rate than enzymatic hydrolysis of the 5'-O-ester ($K_3 >> K_4$). The increased residence time of VEDU in blood samples compared to BEEDU confirmed this postulation.

VEDU has a P value of 1.6 (Keppler et al 1986) which is slightly higher than that for BEEDU (P=1.1). It was previously observed that BEEDU crossed the BBB at a significantly higher ($P < 0.05$) level than EDU. Therefore, it was anticipated that VEDU may also penetrate cells at a level comparable to BEEDU. Injection of [4-^{14}C]-VBEEDU provided significantly higher ($P < 0.001$) radioactivity levels in brain samples compared to that of [4-^{14}C]-EDU (Figure 5.3.3.1). Intravenous injection of [4-^{14}C]-labelled VBEEDU or DVBEEDU provided radioactivity levels equal to 2.5% and 2.2%, respectively, of the injected dose per gram of brain samples taken 3 min post injection. These radioactivity levels are much higher than those after injection of [4-^{14}C]-EDU (0.7%). These higher radioactivity levels were more pronounced in brain samples taken shortly after injection of [4-^{14}C]-VBEEDU. Although, to the best of our knowledge, there are no reported data regarding the effective range of concentrations in brain for treatment of HSV infections in CNS, this observation indicates that VBEEDU crosses the BBB more readily than EDU, most likely due to its higher lipophilicity. However, there was no substantial difference in radioactivity levels in brain samples taken at time period of 3 min and longer post injection of [4-^{14}C]-VBEEDU compared to those of [4-^{14}C]-DVBEEDU. This indicates that a substantial increase in the lipophilicity of prodrugs does not provide a substantially higher radioactivity level in brain, at least, for times > 3 min post injection. The consistently higher radioactivity levels in brain samples taken after injection of [4-^{14}C]-VBEEDU relative to those for [4-^{14}C]-EDU indicate that the prodrug VBEEDU most likely undergoes conversion to EDU. The lower lipophilicity of EDU results in a slower exit from the brain.

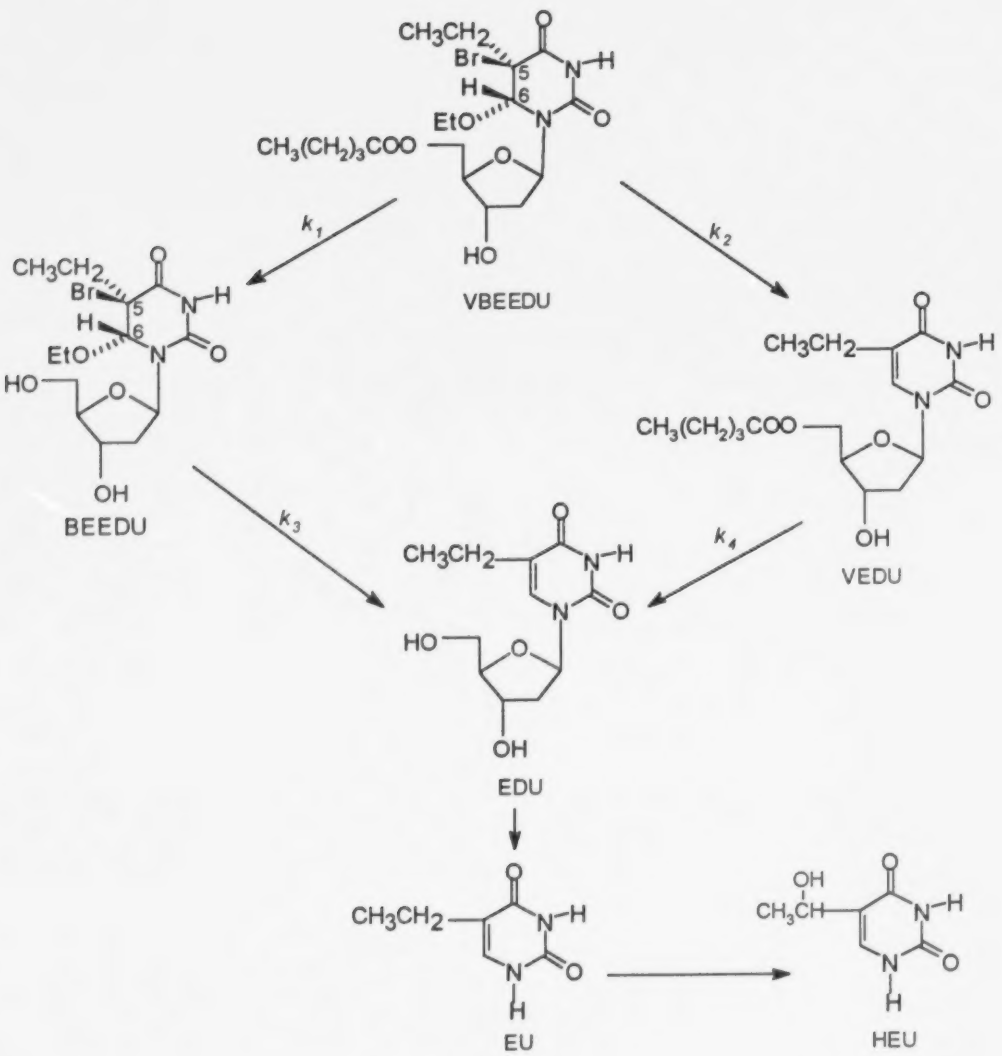


Figure 10.4.5. Proposed mechanisms for the *in vivo* activation of VBEEDU.

In order to evaluate the effect of different routes of administration, VBEEDU and DVBEEDU prodrugs were also administered via the jugular vein into mice. In contrast to BEEDU, brain samples taken after jugular injection of both [4-^{14}C]-labelled VBEEDU and DVBEEDU showed significantly higher radioactivity levels than those after tail vein injection (Figure 11.4.5). Injection of [4-^{14}C]-VBEEDU via the jugular vein provided significantly higher ($p < 0.001$) radioactivity levels in brain samples, with the highest radioactivity level (3.6% of the injected dose) in samples taken 3 min post injection, compared to those after tail vein injection. Jugular vein injection of [4-^{14}C]-DVBEEDU also provided significantly higher radioactivity ($P < 0.001$) levels in brain samples compared to those using the tail vein injection route. The highest radioactivity level in brain samples taken following jugular vein injection of [4-^{14}C]-DVBEEDU was observed in samples taken 3 min post injection (Table 11.3.3.1).

Although the significantly higher radioactivity levels present in brain samples following jugular vein injection of [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU may validate the suggestion that injection of these highly lipophilic prodrugs into a larger vein may increase the percent of the injected dose in brain samples, these results should be interpreted cautiously. It is believed that there are other observations which should be considered before making any conclusion about the effect of the route of administration. First, the test compounds were injected into the jugular vein toward the head of animal (upward), thereby causing additional back-pressure in the venous system. Visual examination of the animal carcass before sampling revealed obvious physical damage and hemorrhage in brain which could be attributed to the method of injection and the close proximity of the injection site to the brain. Therefore, it might be concluded

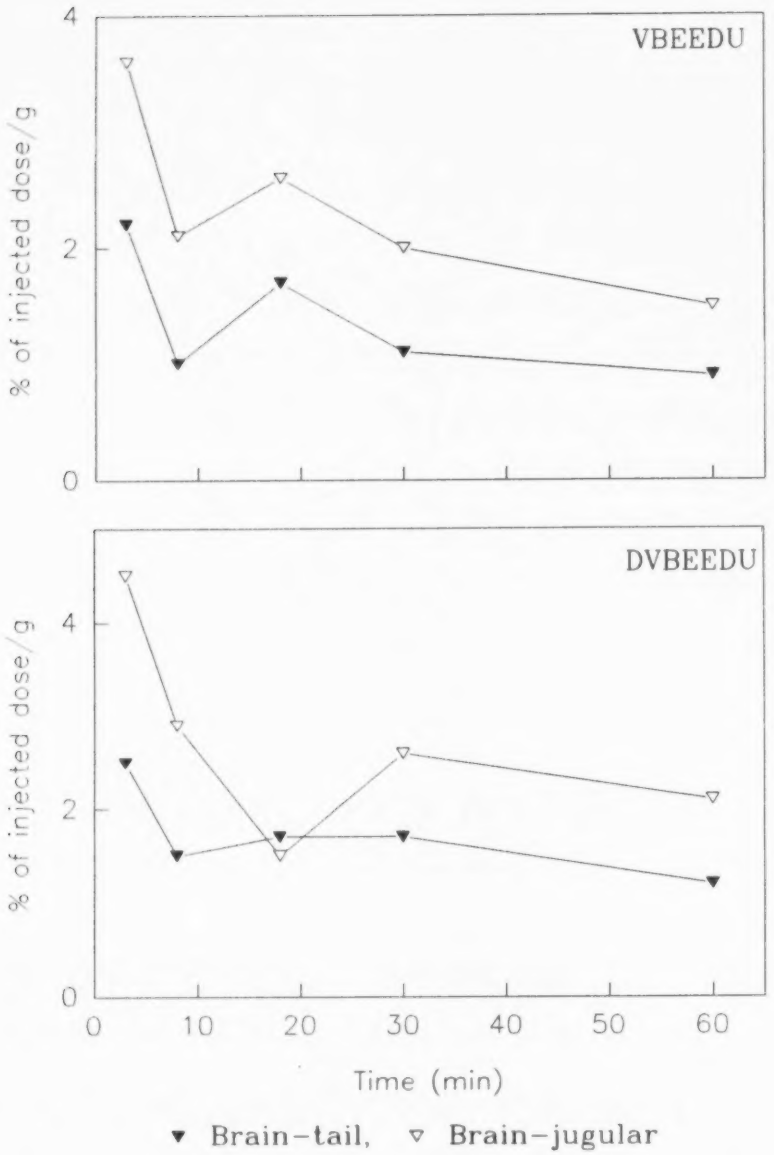


Figure 11.4.5. Radioactivity present in brain samples after injection of [4-^{14}C]-labelled VBEEDU and DVBEEDU via the tail vein and jugular vein. Data are the means of three separate experiments.

that this physical damage and subsequent bleeding cause elevated radioactivity levels due to presence of blood inside the brain samples. With respect to liver uptake, there is no substantial difference in hepatic concentration of compounds whether injected via the tail vein or jugular vein. Therefore, the differences between radioactivity levels of liver samples taken shortly following administration of [4-^{14}C]-BEEDU and [4-^{14}C]-DVBEEDU (section 3.3.2) by these two different routes of injection should be considered cautiously.

However, despite improved pharmacokinetics of EDU, and also significantly higher brain radioactivity levels following injection of BEEDU or VBEEDU, neither EDU nor its prodrugs (BEEDU, VBEEDU) protected NMRI mice inoculated intracerebrally with HSV-1 or HSV-2. This observation differs from previously reported results regarding the efficacy of EDU to protect herpes simplex encephalitic-mice (Davis et al 1978, 1979). However, there were several differences in the experimental protocol used which might have contributed to different results. First, Davis et al reported the number of surviving mice at the 15th day post inoculation, whereas in our study number of surviving mice were counted at the 20th day post inoculation. Second, Davis et al injected the test compound via the iv route, and we injected the compounds via the ip route. Based on high liver extraction and incomplete bioavailability of EDU and its 5,6-dihydro prodrugs, this difference in route of drug administration may influence the survival rate. However, perhaps the most important difference between these experiments is the dose of drug. Davis et al treated animals with a much higher doses (1-8 mmol) of EDU than those we did (0.20-0.78 mmol). It is clear that the inability of BEEDU or VBEEDU to protect HSV-encephalitic mice is most likely due to the fact that the parent compound,

EDU, was not effective against HSE. Based on the *in vitro* activities exhibited by the prodrugs against different strains of HSV, it was not expected that the prodrugs would show a different *in vivo* efficacy against HSV.

It is conceivable that a double prodrug can therefore be designed that possesses physicochemical and/or pharmacokinetic properties that provide a high concentration of the parent nucleoside within a short time period, or act as a sustained releaser of the parent drug. Careful selection of appropriate 5,6-substituents and/or ester substituents present in the sugar ring may provide a double prodrug which would undergo a very rapid cascade of bioactivation reactions to provide a high concentration of the parent compound at the desired site of action, or provide a steady state concentration of the parent nucleoside in blood. A steady state concentration of the antiviral nucleoside could improve its therapeutic efficacy. On the other hand, a prodrug which provides a high concentration of the parent nucleoside within a short time period would also provide a valuable diagnostic tool for early detection of viral infection in the CNS. For example, application of the prodrug model described in this study to radioiodinated IVDU analog, could have diagnostic potential as a non-invasive probe for early diagnosis of HSE using nuclear medicine procedures.

4.6. Oral bioavailability of BEEDU and VBEEDU in mice

EDU showed a 49% oral bioavailability in mice following an oral dose of 0.4 mmol/kg. In order to investigate the effect of 5,6-dihydro prodrugs and also the double prodrug, the oral bioavailability of BEEDU and VBEEDU were investigated in Balb-C mice under similar conditions.

Results of this study were discussed in section 3.8. BEEDU and VBEEDU are biotransformed to EDU after po administration, and that they provided a substantially higher bioavailability for EDU. Oral administration of BEEDU increased the bioavailability of EDU from 49% after administration of EDU to 81%. However, comparison the AUC_{po} of EDU after po administration of VBEEDU, and the AUC_{iv} of EDU after iv injection of EDU, resulted in a 94% (h) oral availability for EDU. The increased oral availability of EDU using BEEDU could be due to the increased lipophilicity of BEEDU. It was previously discussed that the higher lipophilicity of BEEDU (P=1.1), compared to that of EDU (P=0.08), enhanced the cell penetration of BEEDU. In fact, the AUC for EDU after po administration of BEEDU (1.7 ± 0.2 $\mu mol.g^{-1}.min$) was substantially higher than after EDU administration (0.7 ± 0.1$\mu mol.g^{-1}.min$). The half-life and MRT for EDU after either po or iv administration of BEEDU were also higher than those of EDU. The oral availability of EDU after administration of VBEEDU (89%) was similar to that of BEEDU (81%). However, comparison the AUC_{po} of EDU after po administration of VBEEDU, and the AUC_{iv} of EDU after iv injection of EDU, resulted in a 94% (h) oral availability for EDU. The AUC for EDU after po administration of VBEEDU (1.6 ± 0.1 $\mu mol.g^{-1}.min$) was also substantially higher than that for EDU (0.7 ± 0.1 $\mu mol.g^{-1}.min$).

EDU was metabolized to EU after both po and iv administration of EDU, BEEDU and VBEEDU. In contrast, the AUC of EU after po administration of either EDU, BEEDU or VBEEDU was substantially higher than the corresponding values following iv injection. The ratios of the AUC(EU)/AUC(EDU) after po and iv administration of EDU were 1.8 and 0.7, respectively. This ratio, after po administration of BEEDU and VBEEDU, was also higher than that after iv administration. This observation

indicates that EDU, BEEDU and VBEEDU all undergo catabolic degradation by GI tract phosphorylases and/or similar hepatic enzymes before entering the circulation. However, it appears that the extent of phosphoroylsis of BEEDU and VBEEDU in the GI tract is lower than that of EDU, since the differences between the AUC(EU)/AUC(EDU) after po and iv administration of BEEDU and VBEEDU is lower than that of EDU.

The results obtained in this investigation indicate that the oral bioavailability of EDU can be improved significantly using the 5,6-dihydro prodrugs BEEDU and VBEEDU, and that EDU, BEEDU and VBEEDU undergo some glycosidic bond cleavage in the GI tract and/or hepatic presystemic degradation before entering the general circulation. Improving the oral bioavailability of EDU by using the BEEDU and VBEEDU prodrugs described in this study, suggests that these types of prodrugs may also be useful to improve the oral bioavailability of other pyrimidine nucleosides.

4.7. Accumulation of EDU and its 5,6-dihydro prodrugs in murine lung

As discussed in sections 3.3 and 3.9, the 5,6-dihydro prodrugs of EDU investigated in this study showed substantial radioactivity levels in lung samples following injection of [4-^{14}C]-labelled compounds into mice. In this section the possible significance of this observation with respect to their clinical use will be discussed.

Herpes viruses cause a variety of respiratory tract infections in humans (Nahimas et al 1989, Straus 1985, Portolani et al 1993, Inglis 1993). HSV is reported to be the pathogen most frequently isolated from the lungs of patients with severe respiratory distress. A mortality rate between 27-71% was reported for HSV-positive patients with complicated pneumonia

that were being treated with assisted ventilation (Prellner et al 1992). However, it appears that HSV-infection of the respiratory tract in neonates, young adults and immunocompromised adults is more important (Schuller et al 1993, Whimbey et al 1992, Ryan et al 1992, Koskiniemi et al 1989, Nadel et al 1992, Vitale et al 1993). Several cases of pharyngitis associated with HSV have been reported in adults (McMillan et al 1993). In a study involving 43 neonates, it was reported that HSV was the most frequently detected virus in pharyngeal swabs, occurring in one-third of postnatal patients at 2-5 days (Koskiniemi et al 1989). HSV was also reported to be the most frequently isolated pathogen, including bacteria and fungi, in the lungs of patients with severe respiratory distress (Prellner et al 1992).

The distribution of radioactivity after injection of 126 kBq (3.4 μCi) of [4-^{14}C]-EDU showed that the overall radioactivity levels in blood, liver and lung were similar (Figure 13.3.9). Samples taken 3 min after injection of [4-^{14}C]-EDU showed 9.6, 9.7 and 8.1% of the injected radioactivity per gram of lung, liver and mL of blood, respectively. The distribution of radioactivity in lung after injection of [4-^{14}C]-(5S,6S)-BMEDU and [4-^{14}C]-(5R,6R)-BMEDU were substantially different from each other, and also from that of [4-^{14}C]-EDU. The radioactivity level present in lung samples after injection of both [4-^{14}C]-(5S,6S)-BMEDU and [4-^{14}C]-(5R,6R)-BMEDU was substantially higher than that of blood samples. Injection of [4-^{14}C]-BEEDU into mice provided higher radioactivity levels in lung samples compared to that of after injection of [4-^{14}C]-labelled EDU or BMEDU diastereomers. Injection of [4-^{14}C]-BEEDU resulted in localization significantly ($P<0.05$) higher radioactivity levels in lung samples taken 18 min post injection compared to those of blood and liver samples. Intravenous injection of [4-^{14}C]-VBEEDU did not provide higher radioactivity in lung samples than that

of [4-^{14}C]-BEEDU. The radioactivity level in lung samples after injection of DVBEEDU was similar to that of liver samples. However, lung samples showed a considerably higher percent of the injected dose than did blood samples (Table 8.3.3.1).

BEEDU and VBEEDU were selected as representative models of these 5-bromo-5-ethyl-6-alkoxy-5,6-dihydro prodrugs to EDU, since BEEDU showed a higher accumulation of radioactivity in lung compared to the other 5,6-dihydro derivatives evaluated in this investigation. VBEEDU, a novel double prodrug to EDU, was previously found to undergo bioactivation by ester hydrolysis and 5,6-double bond regeneration reactions to give EDU. HPLC analysis of lung samples taken after injection of either EDU, BEEDU or VBEEDU into mice showed that EDU is the major component present in lung samples. The concentration of EDU in lung samples taken 2 min after injection of EDU was substantially lower than that of blood. However, the concentration of EDU in lung samples obtained at longer times was very similar to that of blood samples. In contrast to blood samples, both EDU and EU showed accumulation in lung samples following injection of BEEDU.

A review of relevent papers in the literature reveals that the accumulation of various nucleosides in lung varies substantially from one nucleoside to another. For example, it was reported that 2-8% of the injected dose of 5-halo-1-(2'-fluoro-2'-deoxy-β-D-ribofuranosyl)uracil and 1-(2'-halo-2'-deoxy-β-D-ribofuranosyl)uracil accumulates in the lung of mice following iv injection (Mercer et al 1989,1987, Abrams et al 1986). 5-Halo derivatives of uracil and 2'-bromo-2'-deoxyuridine and 5-bromo-1-(2'-chloro-2'-deoxy-β-D-ribofuranosyl)uracil also showed up to 8% of the injected dose in lung (Lee 1983). However, it was reported that accumulation of (E)-5-(2-iodovinyl)-2'-deoxyuridine and 5-fluoro-5,6-dihydrouracil nucleosides in lung

was less than 1% of the injected dose (Samuel 1985, Visser et al 1988). Acyclovir, which is currently used for the treatment of respiratory tract HSV infections (Ruben et al 1991), showed a slightly lower radioactivity level in lung relative to plasma after administration of [8-^{14}C]-acyclovir (Miranda et al 1981). However, the radioactivity level in lung was substantially lower than that in blood and liver, but higher than that in muscle and heart (Miranda et al 1981). Administration of [2-^{14}C]-5-(2-chloroethyl)-2'-deoxyuridine, which is structurally related to EDU, showed higher radioactivity levels in lung relative to that in liver and blood (Szinai et al 1989). In contrast to other nucleosides, our study and others (Szinai et al 1989) show that EDU and 5-(2-chloroethyl)-2'-deoxyuridine accumulate in lung to a greater extent than blood and liver. More importantly, our study demonstrates that 5-bromo-5-ethyl-6-alkoxy-5,6-dihydro prodrugs to EDU provide a higher concentration of EDU in lung than does EDU itself.

Lung is usually considered as an organ with low metabolic enzyme activities and because it is much smaller than the liver, its role in drug metabolism is usually neglected (Tam 1993). Although the metabolic activity in the lung is much lower than that of the liver, it has been reported that the lung is capable of metabolizing a large number of endogenous, as well as exogenous, compounds. While the lung may not metabolize drugs as efficiently as liver, it is capable of accumulating significant amounts of highly lipophilic drugs such as lidocaine, propronalol and propafenone. Endothelial cells are believed to be responsible for the uptake of these drugs. Highly lipophilic compounds apparently pass quickly into, and through, lipid membranes (Tam 1993, Wilson 1982, Dahl et al 1993)

The 5-bromo-5-ethyl-6-alkoxy-5,6-dihydro prodrugs to EDU, described in this investigation, provided higher radioactivity levels in lung

than in liver and blood after injection of the ^{14}C-labelled compounds. This observation clearly illustrates processes of accumulation and/or metabolic trapping in lung. It was previously shown that these 5,6-dihydro prodrugs are rapidly metabolized to the parent nucleoside EDU. Therefore, it was anticipated that the radioactivity present in lung was primarily due to the presence of EDU and/or EU. It appears that the high radioactivity level present in lung samples after injection of [4-^{14}C]-EDU is mainly due to its rapid biodistribution, where the overall distribution of radioactivity in lung, liver and blood samples was very similar. On the other hand, lung samples taken 8 min after injection of [4-^{14}C]-(5S,6S)-BMEDU and [4-^{14}C]-(5R,6R)-BMEDU showed a high accumulation of radioactivity in lung with T_{max}'s of 18 and 8 min, respectively . It should be noted that the level of radioactivity in lung samples taken after injection of [4-^{14}C]-(5S,6S)-BMEDU was substantially different than that after [4-^{14}C]-(5R,6R)-BMEDU. This observation indicates that the configuration of the 5,6-dihydro prodrug may also play a role in the accumulation of the compound in lung, since (5S,6S)-BMEDU and (5R,6R)-BMEDU are two different diastereomers of 5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine. Lung samples taken after injection of [4-^{14}C]-BEEDU showed a considerably higher percentage of the injected dose relative to those for [4-^{14}C]-EDU, [4-^{14}C]-(5S,6S)-BMEDU and [4-^{14}C]-(5R,6R)-BMEDU. Although the lipophilicity of VBEEDU is 10-fold higher than that of BEEDU , radioactivity levels present in lung samples after injection of [4-^{14}C]-VBEEDU were lower than those for [4-^{14}C]-BEEDU. However, the percentage of the injected dose in lung samples at 3 min post injection of [4-^{14}C]-VBEEDU was significantly higher than that for liver and blood samples. Injection of [4-^{14}C]-DVBEEDU, which is a highly

lipophilic 5,6-dihydro prodrug of EDU, provided significantly higher radioactivity levels in lung samples than in blood, but not in liver samples .

The concentrations of EDU and EU after injection of EDU, BEEDU and VBEEDU were compared in lung and blood samples (Figure 14.3.9). The concentration of EDU in lung was substantially lower than that in blood at early time periods after injection of EDU. In contrast to blood, EU showed accumulation in lung samples, whereas the accumulation of EDU and EU in lung samples, relative to blood, was higher after injection of BEEDU. Lung samples showed a high concentration of EDU shortly after injection of VBEEDU, but there was a rapid decrease 5 min after injection. Lung samples taken after injection of EDU, BEEDU and VBEEDU showed a higher concentration of EU compared to those of blood samples. The higher concentrations of EU, which is a metabolite of EDU, in lung samples compared to blood samples are indicative of pulmonary cleavage of the glycosidic bond in EDU.

A putative mechanism for accumulation of EDU and EU after administration of the 5,6-dihydro prodrugs is described in figure 12.4.7. It appears that the diffusion of EDU, and its 5,6-dihydro prodrugs (P-EDU), into the lung is mainly governed by the lipophilicity of these compounds. Therefore, more lipophilic prodrugs (P-EDU) diffuse into lung more rapidly than EDU ($k_1 >> k_2$). Following their conversion to EDU in lung (k_5), P-EDU provides a high concentration of EDU in lung. However, conversion of P-EDU to EDU in blood (k_4) and subsequent diffusion of EDU into lung (k_2), could also contribute to increasing the amount of EDU inside the lung. The fact that the amount of EU in the lung was much higher than that in blood, after administration of both EDU and P-EDU, indicates that conversion of EDU to EU in the lung (k_7) is more efficient than that in blood (k_6), and also

that EU does not diffuse in and out of the lung (k_3 and k_{-3}) in significant amounts.

At present little is known about the metabolic fate of pyrimidine nucleosides during lung passage. To our knowledge, the kinetic parameters for the elimination of most antiviral and/or anticancer nucleosides by the lung have not been determined. However, there have been studies which investigated the elimination of FUDR from lung tissues. Foth et al reported that pulmonary tissues possess a marked "intrinsic" capacity to transform FUDR into 5-fluorouracil (FU). In contrast, the catabolism of FU and 5,6-dihydrouracil is virtually lacking (Foth et al 1990). It has also been reported that FUDR is converted to FU in human lung tissue (Michihiko et al 1990). In normal lung tissue, FUDR undergoes almost complete glycosidic bond cleavage since the nucleoside phosphorylase activity is much higher than thymidine kinase activity in lung (Michihiko et al 1990). The nucleoside phosphorylase activities in lung, colon and liver tissues were found to be much higher than that of plasma. Michihiko et al therefore suggested that plasma may not be the most important site for FUDR degradation. The results of the current investigation also clearly show that lung is capable of converting EDU into EU.

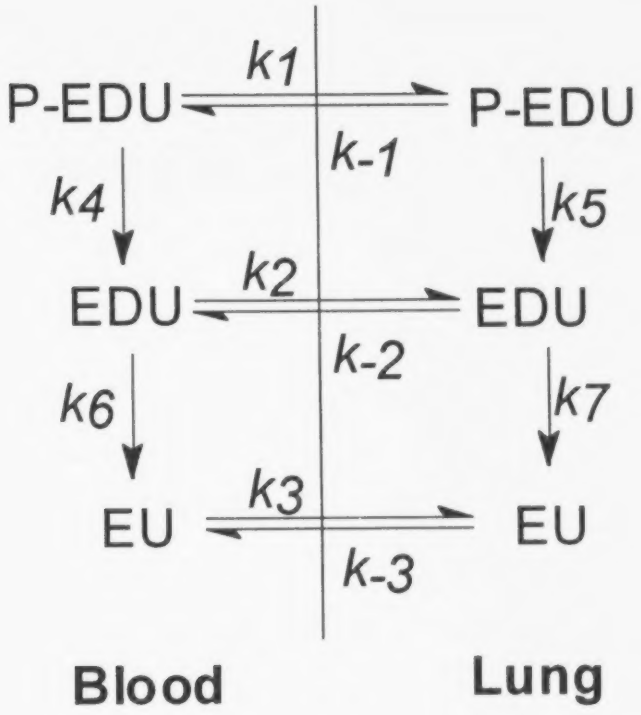


Figure 12.4.7. Putative mechanism for the accumulation of EDU, EU and 5,6-dihydro prodrugs of EDU (P-EDU) in murine lung.

In conclusion, the results of this study show that substantial percentages of the injected dose of [4-^{14}C]-EDU and its 5-bromo-5-ethyl-6-alkoxy-5,6-dihydro prodrugs localize in lung tissues. The accumulation of radioactivity and/or quantity of EDU and/or EU, which are metabolites of these prodrugs, did not correlate with their lipophilicities. Although the lipophilic (5S,6S)-BMEDU, (5R,6R)-BMEDU and BEEDU showed a higher radioactivity level in lung samples after injection of the respective ^{14}C-

labelled compounds, injection of the more lipophilic [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU did not show linear increases in radioactivity present in lung. Although the mechanisms of accumulation have not been resolved, it is concluded that these 5-bromo-5-ethyl-6-alkoxy-5,6-dihydro prodrugs to EDU could serve as good candidates for treatment of respiratory tract HSV-infections.

4.8. Biliary excretion of (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU

It was previously discussed (section 3.3) that the 5,6-dihydro prodrugs of EDU investigated in this study showed a high level of radioactivity in liver, gall bladder, large intestine and small intestine. Since this observation may indicate that entrohepatic recirculation is occurring for these 5,6-dihydro prodrugs, an experiment was designed to establish the biliary excretion of selected 5,6-dihydro prodrugs of EDU, which included (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU. Biliary excretion of some antiviral nuclosides such as carbovir, a carbocyclic nucleoside, was reported previously (Yeom et al 1989, Walsh et al 1990). Following injection of the [4-^{14}C]-labelled compound, all of these 5-bromo-6-alkoxy-5,6-dihydro derivatives of EDU provided a higher percentage of the injected dose in liver than in blood. A substantial amount of radioactivity was also present in large intestine, small intestine and the gall bladder. Intestine samples taken at longer times post injection of the test compound showed a higher percentage of the injected dose than those taken at shorter time intervals.

Biliary excretion of (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU were investigated following injection of the [4-^{14}C]-labelled compound into the jugular vein of rats having an implanted bile duct

catheter. Results from this study were discussed in section 3.10. Bile samples that were collected 8 min post injection of these 5,6-dihydro prodrugs showed the highest radioactivity levels. However, the excretion rates of radioactivity in bile after injection of all four 5-bromo-6-alkoxy-5,6-dihydro prodrugs to EDU were quite similar. Accumulation of radioactivity in bile samples collected after injection of [4-^{14}C]-BEEDU was substantially higher than that after injection of [4-^{14}C]-(5S,6S)-BMEDU, [4-^{14}C]-VBEEDU and [4-^{14}C]-DVBEEDU.

The increased molecular weights of these prodrugs, in conjunction with the presence of polar groups in their structures, may act as driving forces for their excretion into bile. It has been reported that compounds with molecular weights between 300 and 500 are usually excreted in both urine and bile. In addition to a higher molecular weight, drugs that are excreted into bile usually possess a strongly polar group(s) and/or a higher bile/plasma concentration ratio (Shargel et al 1993, Klassen et al 1984, Tse et al 1978).

An initial accumulation of radioactivity in the gall bladder after injection of the [4-^{14}C]-labelled 5-bromo-6-alkoxy-5,6-dihydro prodrugs into mice, and their subsequent secretion into the large and small intestines, would provide a high percentage of the injected dose in intestinal samples. It appears that there is a direct relationship between secretion of radioactivity into the intestine via the bile and the molecular weights of these 5,6-dihydro prodrugs. Among the four 5,6-dihydro derivatives of EDU investigated, (5S,6S)-BMEDU showed the lowest overall radioactivity level in intestinal samples obtained after injection of the [4-^{14}C]-labelled prodrugs.

The radioactivity excretion rates in bile after injection of the four [4-^{14}C]-labelled prodrugs in rats were quantitatively similar (Figure 18.3.10). However, BEEDU showed a slightly higher excretion rate into bile relative to BMEDU, VBEEDU and DVBEEDU. In addition [4-^{14}C]-BEEDU also provided a substantially higher cumulative radioactivity level in bile samples collected up to 4 hr post injection relative to the other three [4-^{14}C]-labelled compounds in rats. There is precedence that conjugated derivatives comprise the major type of products excreted in bile (Shargel et al 1993, Klassen et al 1984). Therefore, the enhanced levels of radioactivity in bile following injection of radiolabelled BEEDU compared to VBEEDU and DVBEEDU, which have higher molecular weights, could be due, at least in part, to the fact that BEEDU has two free hydroxyl groups in the sugar moiety. These hydroxyl groups are readily available for glucuronide conjugation which would afford highly hydrophilic metabolites having a higher molecular weight. These O-glucuronide metabolites would be very good candidates for biliary excretion (Shargel et al 1993, Klaassen et al 1984). The conjugated metabolites are the major metabolites of some of the antiviral pyrimidine nucleosides such as AZT (Restar et al 1989).

Biliary excretion is often an active process that may elevate the blood concentration of drugs through entrohepatic recirculation (Shargel et al 1993). The presence of specific transporters located in the hepatic canalicular membrane, which are capable of transporting purine and pyrimidine nucleosides (Che et al 1992, Moseley et al 1991) and biliary excretion of the antiviral nucleoside carbovir, a carbocyclic nucleoside analog, have been reported (Huang et al 1991, Soria et al 1993). However, it is not clear whether nucleoside transporters present in the hepatic

canalicular membrane play a role in the biliary excretion of this class of antiviral nucleosides.

The total radioactivity in bile (between 0 → 4 hr) following injection of [4-^{14}C]-labelled 5,6-dihydro prodrugs of EDU into rats is summarized in Table 3.4.8.

Table 3.4.8. Total radioactivity excreted in bile during a 4 hr period following injection of [4-^{14}C]-labelled 5,6-dihydro prodrugs of EDU into rats.

Compound	Total radioactivity (% injected dose)
[4-^{14}C]-BEEDU	3.9 ± 0.1
[4-^{14}C]-DVBEEDU	3.2 ± 0.8
[4-^{14}C]-(5S,6S)-BMEDU	2.9 ± 0.8
[4-^{14}C]-VBEEDU	2.9 ± 0.1

[4-^{14}C]-BEEDU showed the highest amount of radioactivity excreted in bile compared to the other prodrug investigated in this study. In contrast to the results observed in mice, total radioactivity excreted in bile for all of these 5,6-dihydro prodrugs is negligible. Therefore, it is very unlikely that this low amount of the prodrug in bile would cause any significance effects in pharmacokinetic of these prodrugs in rats.

4.9. Blood/plasma ratio for the 5,6-dihydro prodrugs of EDU

Based on differences in the biotransformation of BEEDU in whole blood and plasma, it was decided to design an experiment to investigate whether 5,6-dihydro prodrugs to EDU show any accumulation in blood cells. [4-^{14}C]-labelled (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU were injected into rats and radioactivity levels in blood samples were compared with those of the corresponding plasma samples. Results from this study were presented in section 3.11. Blood and plasma samples collected at the same post injection times showed substantially different levels of radioactivity (Figure 17.3.10). Radioactivity levels in blood samples taken at 8 min or longer post injection of [4-^{14}C]-VBEEDU showed significantly ($P < 0.05$) higher radioactivity levels than in the corresponding plasma samples. Although blood samples collected 18 min post injection of [4-^{14}C]-BEEDU showed significantly ($P < 0.05$) higher radioactivity levels than those for the corresponding plasma samples, radioactivity levels in blood samples collected at time intervals shorter than 35 min post injection of [4-^{14}C]-(5S,6S)-BMEDU did not show significant differences compared to plasma samples.

The 5-bromo-6-alkoxy-5,6-dihydro prodrugs to EDU investigated in this study showed substantially different concentrations in whole blood and plasma. Facilated diffusion transporters which concentrate certain nucleosides in red blood cells have been reported (Jarvis et al 1982, Oliver et al 1970, Plagemann et al 1990). It has been reported that several antiviral nucleosides such as AZT, desciclovir and ganciclovir, and also the anticancer drug 5-fluorouracil are transported into blood cells by different mechanisms (Domin et al 1990, Zimmerman et al 1987, Mahony et al 1981,

Domin et al 1990). Although the transport of (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU was not investigated, these compounds are lipophilic prodrugs to EDU which could easily diffuse into blood cells. It is possible that the differences between the radioactivity levels present in blood samples compared to those of plasma samples collected after injection of the [4-^{14}C]-labelled prodrugs (5S,6S)-BMEDU, BEEDU, VBEEDU and DVBEEDU is dependent simply upon the lipophilicity of these prodrugs. The partition coefficient has traditionally been used as an indicator of the ability of a drug to diffuse across a membrane.

Blood samples collected at 18 min and longer post injection times for [4-^{14}C]-BEEDU showed significantly ($P < 0.05$) higher radioactivity levels relative to the corresponding plasma samples. Injection of [4-^{14}C]-VBEEDU, which is the most lipophilic prodrug of EDU studied, also provided a significantly higher ($P < 0.05$) radioactivity level in blood samples compared to the corresponding plasma samples. In contrast, injection of [4-^{14}C]-(5S,6S)-BMEDU did not provide significantly higher radioactivity levels in blood samples collected up to times of 35 min post injection, compared to those for the corresponding plasma samples. It would be expected that lipophilic compounds, which do not undergo intracellular metabolism in blood cells, should give rise to a constant equilibrium between the inside and outside of blood cells. However, it was previously observed that blood cells are capable of transforming BEEDU to EDU by regeneration of the 5,6-olefinic bond present in EDU (section 3.4). This regeneration of the 5,6-olefinic bond affords EDU, which is a more hydrophilic compound than the prodrug, and its further conversion in blood cells may result in trapping of EDU inside the blood cells.

A putative mechanism which may explain the differences between the concentrations of the 5,6-dihydro prodrugs in whole blood and plasma is shown in figure 13.4.9. Based on this mechanism, the 5,6-dihydro prodrugs diffuse into (k_1) and out of (k_{-1}) blood cells based on their lipophilicity. Regeneration of the 5,6-olefinic bond results in production of EDU both inside the cell (k_2) and in plasma (k_4). However, it was previously observed that the conversion of BEEDU into EDU upon *in vitro* incubation with whole blood was more extensive than in plasma ($k_2 >> k_4$). It was also previously reported that most 5-substituted derivatives of 2'-deoxyuridine equilibrate inside and outside the red blood cells by a facilitated diffusion mechanism (Xu 1988). Since it is known that red blood cells are not capable of biotransforming pyrimidine nucleosides, the differences between radioactivity levels show that other blood components may play a crucial role in regeneration of the 5,6-olefinic bond of the uracil ring and further bioconversion of these 5-bromo-6-alkoxy-5,6-dihydro prodrugs to EDU. The importance of other blood components other than red blood cells, such as leukocytes, with respect to the uptake of some drugs such as chloroquine and hydroxychloroquine reported previously (French et al 1987). However, it has recently been postulated that some structurally similar 5,6-dihydro prodrugs to AZT may also bind to red blood cells and therefore cause a difference between the concentrations of the 5,6-dihydro prodrugs in whole blood and plasma (Wang et al).

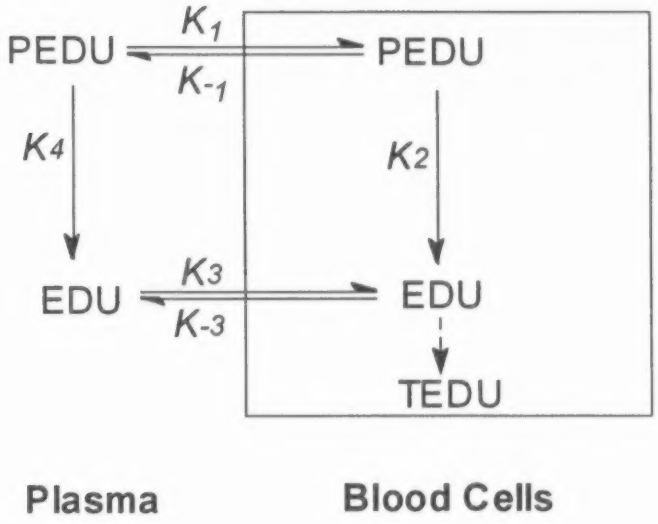


Figure 13.4.9. Putative mechanism responsible for differences between the radioactivity levels of 5,6-dihydro prodrugs to EDU (PEDU) in whole blood and plasma. TEDU is trapped EDU.

Regardless of the exact mechanism for the trapping of EDU in blood cells, this phenomenon causes the trapped EDU to be excluded from the central compartment, thereby precluding its availability to localize in virus-infected cells. The stability of the 5-bromo-6-alkoxy-5,6-dihydro prodrugs, and their rate of conversion to EDU, may play a crucial role in the trapping of EDU, which arises from the prodrug, in blood cells *in vivo*. It was previously stated that (5S,6S)-BMEDU is more stable than BEEDU after iv injection into rats, since (5S,6S)-BMEDU undergoes slower conversion than BEEDU to EDU (section 4.2).Therefore, it was expected that the trapping of

EDU, formed after regeneration of the 5,6-olefinic bond of BEEDU, would be less pronounced at a shorter post-injection times, relative to BMEDU. In contrast to (5S,6S)-BMEDU, blood samples collected 18 min post injection of [4-^{14}C]-BEEDU showed significantly higher radioactivity levels than those of the corresponding plasma samples.

Although the blood/plasma ratio of all of the 5,6-dihydro prodrugs investigated in this study, in most of samples, was lower than two (Table 17.3.11), this ratio was time dependent. It must be recognized that the difference between the concentration of the prodrug in whole blood and plasma would therefore be an important factor when calculating kinetic parameters depending on whether plasma or whole blood were used.

5. Summary and conclusions

The primary objective of this study was to evaluate a series of 5-ethyl-5-halo(Cl,Br)-6-alkoxy(MeO,EtO)-5,6-dihydro-2'-deoxyuridines and (5R,6R)-5-bromo-6-ethoxy-5,6-dihydro-5'(,3')-(di)-O-valeryl-2'-deoxyuridines as potential prodrugs to 5-ethyl-2'-deoxyuridine (EDU) using *in vitro* and *in vivo* models. The following summary and conclusions can be made from the investigations discussed in this thesis.

1. All of the 5,6-dihydro prodrugs to EDU were more lipophilic (P = 0.4-11.5) than EDU (P = 0.08). These 5,6-dihydro prodrugs also showed a higher percent protein binding (%PB) to BSA (11-22%) compared to that for EDU (7%). It was demonstrated that both the nature of the C-5 and C-6 prodrug subsituents, and their configuration, were determinants of lipophilicity and %PB. For example, (5R,6R)-CMEDU (P = 2.5) was more lipophilic than (5R,6R)-BMEDU (P = 1.9) and (5S,6R)-BMEDU (P = 0.4). (5R,6R)-CMEDU also showed higher %PB (22%) than (5R,6R)-BMEDU (19%) and (5S,6R)-CMEDU (11%).

2. In contrast to the (5R,6R)-BMEDU and (5S,6S)-BMEDU diastereomers, the pharmacokinetic parameters for (5R,6R)-CMEDU were different from those for EDU. (5R,6R)-CMEDU undergoes slower clearance (0.7 $L.hr^{-1}$), and it has a longer half-life (2.21 hr) compared to EDU (5.1 $L.hr^{-1}$, 0.69 hr). However, following iv injection into rats, the BMEDU diastereomers undergo more extensive *in vivo* conversion to EDU, compared to the of CMEDU diastereomers.

3. (5R,6R)-CMEDU and (5S,6R)-CMEDU were inactive against HSV-1 and HSV-2 in an *in vitro* assay. However, both (5R,6R)-BMEDU and (5S,6S)-BMEDU exhibited equipotent *in vitro* activity to EDU against HSV-1 and HSV-2. In the HCMV antiviral assay, (5S,6S)-BMEDU was 10-fold less

potent than ganciclovir, whereas (5S,6R)-CMEDU was approximately equipotent to ganciclovir.

4. Metabolic biotransformation of the CMEDU and BMEDU diastereomers, and (5R,6R)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-2'-deoxyuridine (BEEDU), in rats and mice after *in vivo* conversion to EDU was similar to that for EDU. 5-Ethyluracil (EU) and 5-(1-hydroxyethyl)uracil (HEU) were major metabolites of EDU and the 5,6-dihydro prodrugs investigated. In contrast to EDU and BEEDU, 5-(1-hydroxyethyl)-2'-deoxyuridine (HEDU) was detected as a secondary metabolite of both BMEDU and CMEDU diastereomers following iv injection into rats.

5. It was demonstrated that the nature of the C-5 and C-6 prodrug substituents, and their configuration were determinants of the extent of to which the 5,6-olefinic bond was regenerated upon *in vitro* incubation with GSH. In contrast to (5R,6R)-BMEDU and (5S,6S)-BMEDU, BEEDU did not undergo regeneration of the 5,6-olefinic bond upon *in vitro* incubation with GSH. However, BEEDU did undergo regeneration of the 5,6-olefinic bond following a 2 hr *in vitro* incubation with rat whole blood (53%), plasma (8%) and brain homogenate (16%). BEEDU underwent more extensive *in vivo* conversion to EDU following iv injection into both rats and mice. It was concluded that regeneration of the 5,6-olefinic bond upon *in vitro* incubation with GSH may not reflect the *in vivo* behavior of the 5,6-dihydro prodrugs.

6. In contrast to (5R,6R)-BMEDU ($P > 0.05$) and (5S,6S)-BMEDU ($P > 0.05$), BEEDU ($P < 0.05$) and its 5'-O-valerate (VBEEDU) ($P < 0.001$) and the 3',5'-di-O-valerate (DVBEEDU) ($P < 0.001$) esters provided significantly higher radioactivity levels in brain samples following iv administration of the [4-^{14}C]-labelled compounds compared to that of EDU. However, compared to [4-^{14}C]-VBEEDU, [4-^{14}C]-DVBEEDU did not provide substantially higher

radioactivity levels in brain samples taken > 3 min post iv injection into mice.

7. The bioavailability of EDU in male Balb-C mice was increased about 2-fold (81%), compared to that of EDU (49%), using the BEEDU prodrug. However, with respect to pharmacokinetic parameters, VBEEDU did not show any advantage over BEEDU. Oral administration of the VBEEDU into mice provided a 89% bioavailability for EDU. Following oral administration into mice, both EDU and its 5,6-dihydro prodrugs (BEEDU and VBEEDU) undergo glycosidic bond cleavage in the GI tract and/or liver before entering the circulation.

8. Both BEEDU and VBEEDU exhibited a similar *in vitro* potency to EDU against several strains of HSV-1 and HSV-2 . However, EDU, BEEDU, or VBEEDU did not protect NMRI mice inoculated intercerebrally with HSV-1 or HSV-2.

9. EDU, and the 5,6-dihydro prodrugs investigated (BEEDU and VBEDDU), attained a high concentration in murine lung. Lung samples taken at 3 min post injection of [4-^{14}C]-labelled EDU, (5S,6S)-BMEDU, (5R,6R)-BMEDU, BEEDU, VBEEDU and DVBEEDU into mice showed 9.6, 7.4, 11.4, 15.9, 11.6 and 19.8% of the injected dose per gram of lung, respectively. Lung samples taken after iv injection of EDU, BEEDU and VBEEDU into mice showed a higher concentration of EU, a metabolite of EDU, compared to that in blood samples. It was concluded that the higher concentration of EU in lung samples, compared to those of blood samples, is indicative of pulmonary cleavage of the glycosidic bond present in EDU.

10. Injection of [4-^{14}C]-labelled EDU, (5S,6S)-BMEDU, (5R,6R)-BMEDU, BEEDU, VBEEDU and DVBEEDU into mice provided between 0.8-8.1% of the injected dose per 100 mg of gall bladder, and 1.9-5.8% of the

injected dose per gram of small intestine samples taken at 30 min post injection. However, it was observed that < %4 of the iv dose was excreted into bile during a 4 hr period following iv administration of [4-^{14}C]-labelled BEEDU, VBEEDU, DVBEEDU and (5S,6S)-BMEDU to rats.

11. Intravenous injection of [4-^{14}C]-labelled BEEDU, VBEEDU, DVBEEDU or (5S,6S)-BMEDU into rats provided higher radioactivity levels in the majority of whole blood samples compared to those of the corresponding plasma samples. However, it was demonstrated that the whole blood / plasma radioactivity level ratios were time dependent, and that this ratio, for most of samples, was lower than 2 for all of the prodrugs [BEEDU, VBEEDU, DVBEEDU and (5S,6S)-BMEDU] investigated.

Based on the results from the *in vitro* and *in vivo* studies using the selected 5,6-dihydro prodrugs of EDU described in this thesis, it was demonstrated that the 5,6-dihydro prodrug model improved the pharmacokinetic parameters, and/or brain delivery of EDU. It is concluded that the 5,6-dihydro prodrug approach described could serve as a useful method to improve the *in vivo* characteristics of anti-herpes uracil nucleosides.

6. References

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7. Apendix 1. Surgical operation protocols for catheterisation of jugular vein and bile duct in rats.

HEALTH SCIENCES LABORATORY ANIMAL SERVICES
TECHNICAL S.O.P. #017
Non-Survival Jugular Catheterization in Rats (Wiebe)

This technique is used by Dr. Wiebe's lab for pharmacokinetic studies to sample blood, inject substances and euthanize the rat at the termination of the experiment. After sampling the cannula should be flushed with heparinized saline to avoid clotting.

MATERIALS:

a) Instrument Pack
   - scissors (2)
   - tissue forceps (2)
   - suture material

b) Masking tape or plexi-glass board & elastics

c) Anesthetic (Methoxyflurane)
   (Sodium pentobarbital - Somnitol)
   - nose cone
   - knock down jar
   - fume hood

d) 0.56 mm i.d./0.60 mm o.d. catheter with 0.5 cm of 0.02 mm silastic tubing attached.

e) #22 scalpel blade

f) 25G needles and 1 ml syringes

g) gauze sponges

TECHNIQUE:

a) Prepare site and materials (as above).

b) Anesthetize the rat.
   1. If using Methoxyflurane the use of a fume hood or gas scavenger is mandatory as methoxyflurane has been shown to be hepato-toxic. The rat is to be placed in a knock down jar and then maintained in surgical plane anesthesia using a nose cone.
   2. If using sodium pentibarbital, use a 25G needle to inject the rat by the interperitoneal route. Dosage: 30-50 mg/kg BWT.

   As the experiments will require approximately 6 hours of surgical plane anesthesia, if you are using the sodium pentobarbital anesthasia method you will have to supplement the animal to maintain anesthesia after about three hours. This can be done using an inhalation anesthetic (methoxyflurane) to maintain anesthesia, or by topping up the animal with maintenance doses of sodium pentibarbital.

c) The animal is to be shaved as in diagram (A). This is best accomplished by first wetting the area with a mixture of soap and water and using short quick strokes with a #22 scalpel blade to shave the site. **DO NOT SOAK THE ANIMAL AS IT MAY INDUCE HYPOTHERMIA.**

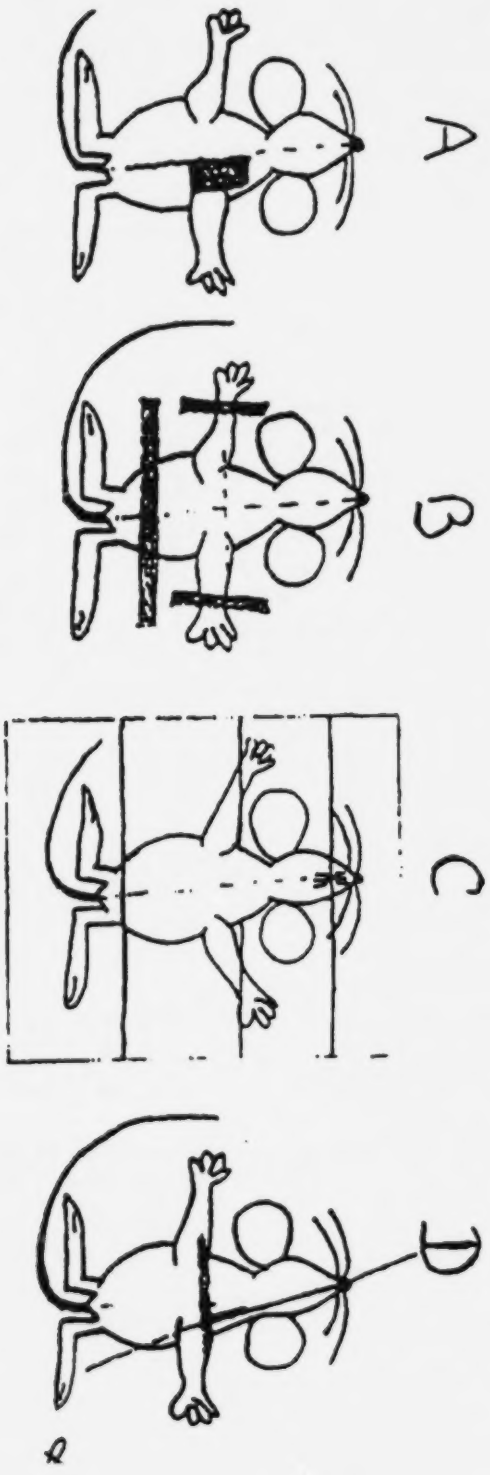
d) The animal is to be secured on the bench in dorsal recumbency with masking tape or by using a plexi-glass board as per diagrams (B) & (C). It is important that the rat is positioned with its vertebrae as straight as possible and the front limbs at 90 degrees to the spine.

e) The animal should be **constantly monitored** using the toe pinch (pedal) reflex to ensure that it is maintained in surgical plane anesthesia.

f) An incision of approximately 0.5 - 1.0 cm is made through the skin. If one was to draw a straight line between the tip of the lower jaw and the first nipple (as located on a female rat), the incision should start at the edge of the pectoral muscle and move toward the jaw, as per diagram (D). Using blunt dissection work your way through the fat and fascia until the pectoral muscle is well exposed and the jugular bulges at the cranial border of the muscle. The placement of a needle sheath under the base of the neck will help to visualize the jugular.

g) Insert the catheter into the vein and secure it using suture. A pair of forceps is used to insert a double threaded suture under the vein. The threads are cut to give two (2) threads. The vein is opened using a pair of fine scissors, and once the catheter is inserted into the vein, the threads are secured on either side of the catheter.

i) At the end of the experiment, ensure that the animal is humanely euthanized by an overdose of sodium pentobarbital given into the jugular catheter. Ensure there are no signs of life and incinerate body.


A
B
C
D

Bile Duct Cannulation

Purpose of this experiment is the simultaneous determination of the concentrations of I.V. injected test compounds in blood and bile.

Three rats will be used for each test compound. Each rat will undergo jugular vein catheterization and bile duct cannulation. The rat will be anaesthetized by injection of pentobarbital by the intraperitoneal route 60 mg/kg (prn 4h). Animals will be completely unconscious before doing any surgery; they should not respond to any pain stimulation (e.g. squeezing its paws, or exciting its ear with needle). This level of surgical anesthesia usually occurs about 10-15 min. after i.p injection of pentobarbital.

For bile duct cannulation, the rat is placed on its back with its tail towards the investigator. A midline abdominal incision is made (about 3 cm). After locating the bile duct by gently lifting the liver lobe with a blunt probe and putting traction on the upper small intestine with blunt forceps, forceps are carefully pushed through the connective tissue under about 0.5-1 cm of duct near the hilum of the liver. This length of bile duct is freed of connective tissue, to expose it for cannulation.

The upper part of this section of bile duct is partially transected with fine scissors, and a 0.28 mm i.d. and 0.61 mm o.d. catheter is pushed into the duct towards the liver (about 4-5 mm). The catheter will be fixed to the bile duct by tying with surgical thread. After securing the catheter, the abdominal incision may be closed or covered with surgical gauze impregnated with petroleum jelly.

Catherterisation of the jugular vein will be performed according to the previously described and approved protocol. Throughout the experiment the rat should be kept in deep anesthesia; a maintenance dose of pentobarbital may be injected, if necessary. The experiment may take up to 4 hr and at the end of each experiment the rat will be sacrificed by injection of an overdose of pentobarbital into the heart (via the jugular vein catheter). The carcass will be disposed of in the proper manner (incineration, or storage in a deep freeze for appropriate periods if the test compound is radioactive).